BASIC PRINCIPLES OF IDENTIFICATION OF MOULDS, YEASTS AND BACTERIA

INSTRUCTIONS AND WORKBOOK FOR FOOD MICROBIOLOGY LABORATORY EXERCISES

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PHOTOS OF MOULDS MICROCULTURES

Microculture 1: *Aspergillus*

Microculture 2: *Chaetomium*

Microculture 3: *Cladosporium*

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INSTRUCTIONS FOR SAFE LABORATORY WORK

- In the microbiological laboratory, always wear lab coats.
- In the microbiological laboratory smoking, eating and drinking is prohibited.
- Only material that you need to carry out an investigation should be on lab worktop.
- Windows and doors must be closed during the experimental work due to the risk of contamination.
- Always use aseptic technique.
- Lab gas burners should be on only when working.
- Work carefully at the lab gas burner.
- If you come to direct contact with sample or microorganisms immediately report to assistant or technician.
- If there is contamination of working environment, it should be disinfected, washed with tap water and wiped with a paper towel.
- Contaminated glassware and plastic tips for automatic pipettes should be placed in prepared table-basket or container with disinfectant.
- Trash that is contaminated with microorganisms should never be placed in waste container.
- Microorganisms should not be moved from the microbiological laboratory.
- Work carefully with microscope; make sure that the microscope light is turned off and cleaned at the end of work.
- Microscope slides should not be throwing in waste container, but in prepared dish with disinfectant.
- After finishing work clean and disinfect lab worktop.
- After finishing work, disinfect hands, wash them thoroughly with soap and water and dry with a paper towel.
- Always work with harmful and toxic substances in a fume hood.
- Write the results of the observations and in the workbook.
INTRODUCTION

Taxonomy is defined as science of biological classification (Greek *taxis* means classification, arrangement, *nomos* means law, principle, rule, *nemein* means arranged). In a broader sense taxonomy contains three areas: classification, nomenclature and identification. The classification means the arrangement of organisms into groups (*taxon*, plural *taxa*) and is based on common characteristics or evolutionary relatedness. Nomenclature deals with allocation of names of taxonomic groups in accordance with published rules (i.e. International Committee on Systematic Bacteriology). Identification is the procedure by which organism/isolate is ranked in group to which isolate belongs. Microbial taxonomy means grouping of microorganisms that have similarities with respect to the specified criteria or characteristics.

**Taxonomic groups, nomenclature**

Basic taxonomic group is species (sp., plural spp.). In eukaryotes the species is often defined as a group of related organisms in which two individuals are capable of reproducing fertile offspring, typically using sexual reproduction. **Bacterial species** is a group of strains that have a common set of stable characteristics and differ from other groups of strains. The strain is a population of organisms, resulting from a single organism. Strains within a species can be distinguished by different properties. A more precise definition of bacterial species is based on the sequences of the ribosomal RNA (rRNA): **Bacterial species is defined as group of prokaryotes, which have 97% or more sequence identity of 16S rRNA**.

By preparation of classification scheme specified microorganism is placed in small homogeneous group, which is part of a larger group. Groups are arranged hierarchically and do not overlap. The most commonly used taxonomic levels are: species, genus, family, order, class, phylum, kingdom and domain.

The name of the microorganism is always made up of two names – it is **binomial system** named after the Swedish botanist Carl von Linne. The first name is the name of genus, which is always written with a capital letter and then the name of the species, which is always written in lowercase. They are
always written in italics or underlined. Genus name may be abbreviated, while the name of the species should never be shortened. Description of individual bacteria and their classification (including a list of recognized names) are given in the Bergey Manual (1994).

**Classification system**

Classical classification of bacteria is based on their **phenotypic properties**. Evolutionary relationship or relationship of prokaryotes is based on **genotypic characteristics**. Classification, therefore, means the distribution of organisms into taxonomic groups (**taxon**) and reflects their degree of relatedness. The hierarchy of groups shows phylogenetic or evolutionary relationships between organisms. Therefore, we are talking about **phylogenetic or evolutionary classification system** (phylogenetic means starting from a common ancestor), which is based more on the evolutionary relationships rather than on the general similarities (phylogeny: evolutionary development of the species; Greek *phylon* means a genus, classified in genus, *genesis* means progeny, origin, genus).

Prokaryotes and eukaryotes are according to the rRNA sequences classified into seven kingdoms, which are shown in Figure 1.

**Figure 1**: Kingdoms of prokaryotes and eukaryotes

(http://www.mycolog.com/CHAP1.htm)
Fungi

Fun
gy in the Eumycota kingdom are according to the way of formation of sexual spores divided into five main phyla and one phylum in which are moulds, which do not form sexual spores. Moulds, which are as important for food spoilage and mycotoxins, are located in three orders as Zygomycota, Ascomycota and Deuteromycota.

Zygomycota

Most of mould in this order belongs to the Zygomycetes class. The main characteristics of the mould in this class are:

- Formation of zygospore: all moulds in Zygomycetes form sexual spores named zygospores (teleomorph) in a diploid reproductive stage. Zygospores are usually large (diameter is usually larger than 30 µm) and dark coloured parts of moulds.
- Rapid growth: Isolates grow very rapidly and in a petri dish with malt extract agar, in 2 to 4 days grow large, fluffy colonies.
- Non-septate mycelium: Mycelium of actively growing mould is without septa (transverse cell walls), which allows rapid movement of protoplasm and its contents (cell nucleus, mitochondria, nutrients) and the formation of spores.
- Reproduction with sporangiospores (anamorph): Sporangiospores are characteristics way of asexual reproduction of moulds in Zygomycetes class. They are asexual spores that are formed in sporangium with collumela or sporangium without collumela on specialised hypha named sporangiophore. Sporangiospores may be formed also in merosporangium which has no collumela. Merosporangium is cylindrical sporangium that has manj mespores. Sporangiospores are formed very rapidly.
- Formation of chlamydospores (another anamorph shape of moulds) some fungi form chlamydospores which are cylindrical to spherical shape with...
relatively thick wall, and is formed in the substrate mycelium. Most of them are more resistant to light, heat and drying as sporangiospores.

Most of moulds in Zygomycetes do not form mycotoxins. They are found in the soil, manure, and they are often as pathogenic moulds on various insects. As food-spoilage moulds are usually of the Mucorales order. They appear mainly on fresh foods with high $a_w$ (water activity) value; they are not resistant to the heat treatment of foods and chemical food preservation.

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**Figure 2: Zygomycota:** A: sporangium with sporangiospores at *Mucor*, B: sporangia at *Absidia*, C: zygospore at *Zygorhynchus*, D: sporangiophore at *Cunninghamella*

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**Ascomycota in Deuteromycota**

Moulds from Ascomycota and Deuteromycota differ from Zygomycota moulds by basic properties and among which the most obvious is formation of **septate mycelium**. The consequence is generally slower growth of these moulds.

**Ascomycota**

Moulds from **Ascomycota** reproduce by sexual spores – **ascospores** that are formed in *ascus* (S: ascus, P: asci). Hyphae have numerous **septa**. Asexual reproduction is often by **conidia**. Most of the Ascomycetes are found in the soil and some in fresh and marine waters.
Deuteromycota

A sexual form of certain mould or structure forming the asexual spores is called anamorph; a sexual form is called teleomorph and together they form a mould in its entirety - holomorph.

Many molds are known only as anamorph and cannot be classed as Ascomycetes or Basidiomycetes to which they may belong. The classification system before 2013 allowed for moulds special designation for anamorph forms and as a consequence lot of moulds have two different names. For example *Eurotium repens* name refers to holomorphic mould with conidia and ascospores incidence, while *Aspergillus repens* is only anamorphic form of the same mould.

Moulds in Deuteromycota are described as moulds with sepaate mycelium that do not form sexual spores. These moulds are also called "Fungi imperfecti" as moulds that have only asexual spores. After mitotic division of nucleus asexual spores are formed singly or in chains on more or less specialized structures and are commonly referred to as conidia (S: conidium, P: conidia). For certain genera are typical formation of conidia on the specialized structure called phialide or in the cell, which is called anelide. Phialide may be located directly on conidiophore, or on conidiophore with additional specialized cells called metulae (S: metula, P: metulae) and phialide that form conidia. Conidiophore is a term that includes air mycelium and metulae and phialide in cases where they are present.
The main characteristics of moulds in Deuteromycota:
• they do not have sexual mode of reproduction,
• most of them are similar to moulds in Ascomycota,
• there are more than 1,680 genera and 17,000 species
• the vast majority of these moulds are found on or in the soil and are saprophytes (causing decay) or parasites (an organism that lives in or on another organism - the hosts and the benefits from it, the host does not) for plants, relatively few animal parasites,
• asexual way of reproduction is most often by spores, which are very varied according to their colouring, surface relief, size, shape, cell number, arrangement and depending on the way to the formation of mycelium - the majority of these properties are used in the identification of genera and species.

Figure 4: Deuteromycota: A: apex with phialide and single-cell conidia at Aspergillus, B: single-cell conidia arranged in tufts on conidiophore at Botrytis, C: multicellular sickle like formed macroconidia at Fusarium, D: anelide and single-cell conidia with truncated blunt end at Scopulariopsis

Glossary of Mycological Terms http://www.mycology.adelaide.edu.au/virtual/glossary/
CLASSIFICATION OF MOULDS

Classification and identification of moulds should be based on a variety of their respective modes of sexual reproduction. Often this is not possible because moulds are forming only asexual spores (anamorph). Moulds, which are as important for food spoilage, are located in three phyla: Zygomycota, Ascomycota and Deuteromycota.

Kingdom: FUNG I

Phylum: CHYTRIOMYCOTA
Phylum: OOMYCOTA
Phylum: ZYGOMYCOTA

Class: ZYGOMYCETES

Order: MUCORALES
Family: MUCORACEAE
Genus: Rhizopus
Genus: Mucor
Family: THAMNIDIACEAE
Genus: Thamnidium

Phylum: ASCOMYCOTA

Class: ASCOMYCETES

Order: SPHAERIALES
Family: SORDARIACEAE
Genus: Neurospora*
Family: EUROTIACEAE
Genus: Byssoschlamis
Genus: Chaetomium

Phylum: BASIDIOMYCOTA
Phylum: **DEUTEROMYCOTA**

Class: HYPOMYCETES

Order: HYPOMYCETALES

Family: DEMATIACEAE
Genus: *Alternaria*  
*Cladosporium*  
*Curvularia*

Family: MONILIACEAE
Genus: *Acremonium*  
*Aspergillus*  
*Botrytis*  
*Geotrichum*  
*Chrysonilia* *  
*Penicillium*  
*Scopulariopsis*  
*Trichothecium*  
*Trichoderma*

Family: TUBERCULARIACEAE
Genus: *Fusarium*

Class: COELOMYCETES

Order: SPHAEROPSIDALES

Genus: *Phoma*
DESCRIPTIONS OF THE MAIN MOULDS

*Mucor*

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is coloured brown to black.
- Aerial mycelium is in the early phase of white color, then gray, and then black and blue green to dark brown, and has low compact structure.

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is aseptate and may be branched. Chlamydospores are formed in substrate mycelium at some species.
- Hyphae are not coloured or lightly colored.
- From hyphae sporangiophores grow and they are as single or in pairs; sporangiophore is aseptate.
- Sporangiophore expands into pear-like columella and around columella is round sporangium. Sporangium has sporangiospores that are oval or round and have smooth or prickly surface (but not furrowed surface as it is at *Rhizopus*).

SIGNIFICANCE:

In/on food at least 20 different species of *Mucor* were found. Some species can grow under anaerobic conditions, thus causing spoilage of drinks (weak fermentation). Growth (such as chains related budding cells) is similar to yeast (cells are much larger than the yeast). Similarly moulds of *Mucor* can grow in substrates with a high concentration of NaCl. Moulds of *Mucor* cause spoilage of cheese, marmalade, soy, rice, corn, potatoes.

PICTURE:

Magnification of microscope:
1. Aseptate substrate mycelium, which might has chlamidiospores
2. Aseptate sporangiophore
3. Columella
4. Round sporangium
5. Sporangiospores
Rhizopus

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium brown.
- Aerial mycelium is gray to brownish black, tall, fluffy structure and compact. Mould grows very quickly. Sporangia are initially white, with a maturation become black.

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is aseptate and has rhizoids, which look like a kind of roots after which the genus has name.
- At the opposite direction of rhizoid sporangiophore is formed which is also aseptate.
- Rhizoids and sporangiophores are usually coloured.
- Sporangiophore is ended with bear like form named collumela. Around collumela is sporangium and in sporangium are sporangiospores. Sporangiospores have often furrowed surface. Columella and sporangium collapses with aging in the form of an umbrella.

SIGNIFICANCE:

Moulds of Rhizopus cause rot on different fruits (berries) and vegetables (beans, peas, cauliflower). Some species are industrially important (R. oligosporus) at different fermentations.

PICTURE:
Magnification of microscope:
1. Aseptate substrate mycelium
2. Rhizoids
3. Aseptate sporangiophore
4. Collumela
5. Sporangium with sporangiospores
6. Round and oval sporangiospores
**Chaetomium**

**MACROMORPHOLOGICAL PROPERTIES:**
- Substrate mycelium may be colorless, brown, gray or gray-black.
- Colonies are fast growing, at first white, then gray color to olive green to greyish-brown or black-brown color due to the formation perithecia, giving the typical colony appearance. Aerial mycelium has a fluffy structure.
- Differentiating species of Chaetomium:

<table>
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<tr>
<th>SPECIES / MEDIUM</th>
<th>C. brasiliense</th>
<th>C. funicola</th>
<th>C. globium</th>
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<td>Aerial mycelium on CYA</td>
<td>25-30 mm, white to gray</td>
<td>20-25 mm, low white mycelium sometimes by gray patches</td>
<td>low scarce white mycelium with rare black perithecia (2r=0,2 mm)</td>
</tr>
<tr>
<td>Substrate mycelium on CYA</td>
<td>dark gray to black, sometimes colorless</td>
<td>colorless or gray-brown colour</td>
<td>brown substrate mycelium</td>
</tr>
<tr>
<td>Aerial mycelium on MEA.</td>
<td>25-30 mm, temno sive barve</td>
<td>35-40 mm, low colonies of white or gray</td>
<td>dense growth, low, gray to gray-black, enveloping hyphae many peritecia</td>
</tr>
<tr>
<td>Substrate mycelium on MEA</td>
<td>temno sive barve</td>
<td>colorless to gray or light brown mycelium</td>
<td>brown substrate mycelium</td>
</tr>
</tbody>
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**MICROMORPHOLOGICAL PROPERTIES:**
- Perithecium is an enclosed ascocarp characterized an apical ostiole and by asci arranged in a basal tuft or hymenium layer. They are dark brown to black, and has shape that look like egg or flat bottle with long dark coloured sterile hypae. Hypae are septate and unbranched by a rough surface (C. globsum)
- Perithecia have an opening – ostiole, through which arising ascus or ascospores. In perithecium are asci that are cilindrical shape. In asci are 4-8 single cell ascospores tah are olive brown and have shape like lemon.

**SIGNIFICANCE:**
Moulds of Chaetomium have strong cellulase activity and are often in the substrate with a large cellulose, for example, in soil, manure and rotting vegetation, materials with a high moisture content (for example, wood, textile, paper).

**PICTURE:**
Magnification of microscope:
1. Perithecium with hyphae
2. Septate hyphae
3. Ascospores in shape of lemon
**Acremonium**

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is uncoloured or light beige to light ocher colour.
- Aerial mycelium is low, filamentary structures, light-colored from white to bright pink, bright orange, yellow, green or olive green. Colonies are low, a very slow-growing and have a diameter of less than 25 mm (MEA, incubation at 25°C for 10 days).

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium septate and thin. On the microscopic slide entanglements hyphae are seen.
- Conidiophores arising from substrate mycelium, they are aseptate and very short.
- Conidiophores form long phialide. For certain types of phialide grown on the substrate mycelium, Phialide are simple and usually have a form of awl.
- Phialide form conidia sequentially, but not in chain. Conidia are unicellular, elongated cylindrical or oval in shape; they may also be non-symmetrical shape. Conidia are in groups, often combined in an adhesive heads.

SIGNIFICANCE:

In *Acremonium* genus are for food important food three species: *Acremonium butyri*, *Acremonium strictum* in *Acremonium charticola*. In nature, these moulds are widespread as saprophytes, *Acremonium charticola* can be pathogenic for humans. They are causing rot on apples, pears, bananas and fresh vegetables. *Acremonium strictum* are often isolated from wheat, rye and rice.

PICTURE:
Magnification of microscope:
1. Substrate mycelium: septate, thin
2. Conidiophore: short and aseptate
3. Conidia:
   - unicellular, round to cylindrical,
   - grouped
**Alternaria**

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is brown black.
- Aerial mycelium is low, compact, dirty grey to green in colour, or a grey-black in colour, the colonies are low and compact and have a diameter of 50-60 mm (CYA, incubation at 25°C for 7 days).

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is septate with 1 to 3 septa, and it is very thin. On microscopic slide substrate mycelium is lighter and thinner than conidiophore.
- From hyphae grow conidiophores that are septate and short (one, two or three cells). On microscopic slide they are usually broken and darker than the substrate mycelium.
- Conidiophore forms conidia. In the early phase conidia are unicellular and conical. With maturation conidia become septate, first with transverse septa, then longitudinal septa and they have characteristic shape of a club. Conidia are often linked in chains.

SIGNIFICANCE:

In *Alternaria* genus are described several species and most are pathogenic to plants like wheat, cabbage, broccoli, cauliflower and other vegetables, as spoilage these moulds are located on melons and citrus fruits. Individual species form different toxins and secrete them in foods (tomatoes, wheat, corn, olives).

PICTURE:

Magnification of microscope:
1. Substrate mycelium: septate, thin
2. Conidiophore: septate, short
3. Conidia:
   - unicellular,
   - with transversal and longitudinal septa
**Aspergillus**

MACROMORPHOLOGICAL PROPERTIES:
- Substrate mycelium: *A. flavus* yellow, ocher to brown pigments; *A. niger* yellow to ochre pigments.
- Aerial mycelium: *A. flavus* has green to olive yellow aerial mycelium, colonies have a diameter of 60 – 70 mm (CYA, incubation at 25°C for 7 days), they are low and the structure of cotton wool. *A. niger* has white low aerial mycelium and black conidia with diameter of 60 mm or more (CYA, incubation at 25°C for 7 days).

MICROMORPHOLOGICAL PROPERTIES:
- Substrate mycelium is septate, much thinner than air mycelium and branched.
- Conidiophore is not septate, it is much stronger, thicker than the substrate mycelium and at top is extended in apex. Basal cell in substrate mycelium, conidiophore and apex form one cell.
- Phialide or phialide and metula are formed on apex, phialide are radially arranged. There may be only primary phialide or primary and secondary phialide what depends on *Aspergillus* species. Phialide grow simultaneously, while at *Penicillium* phialide grow rastejo gradually. For *A. flavus* are typical that have metula, for *A. niger* phialide and metula.
- Conidia are formed on phialide. Conidia are round with smooth or a little rough surface, and in chains of 50. Around conidia is never envelope!

SIGNIFICANCE:
Among more than 150 known species of *Aspergillus* genus, 30 species is described in details and of these approximately 21 species are found in food. *Aspergillus* moulds are among the most common food spoilage and together with moulds from *Penicillium* and *Fusarium* are dominant group. They cause spoilage of peanuts, hazelnuts, rice, cereals and cereal products, cold meats, cheeses and other fruits and vegetables. Some species (e.g. *A. flavus*) form aflatoxins, which are the most important mycotoxins. Food that is contaminated with *Aspergillus* is not suitable for use.

PICTURE:
Magnification of microscope:
1. Tin, septate substrate mycelium
2. Aseptate conidiophore
3. Apex with flask-shaped phialide
4. Conidia: round in chains
Botrytis

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is white to grey.
- Aerial mycelium has filamentary structures, at the first is white, and later grey to dark grey.

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is septate.
- It has long conidiophore, which is septate and at the end asymmetrically branched.
- Conidia are unicellular, ellipsoidal, and typically arranged in tufts on conidiophore.

SIGNIFICANCE:

As a pathogenic fungus Botrytis occurs in many plants. Particularly susceptible to rotting are vegetables (onion, tomato), strawberries and other fruits (grapes, apples, pears, strawberries, kiwi), an infection can occur prior to the maturity of the fruit, or later during storage and processing. As spoilage microorganism also occurs in various food products.

PICTURE:
Magnification of microscope:
1. Substrate mycelium: septate
2. Conidiophore: septate, asymmetrically branched
3. Conidia: unicellular, ellipsoidal, and typically arranged in tufts
**Chrysonilia**

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is colourless.
- Aerial mycelium has the cotton wool and filamentary structures, from white to pink colour and very quickly grow entire medium (three days) and it forms a large orange conidia, which are dangerous to laboratory contamination. Therefore, it is preferable to cultivate *Chrysonilia* in tubes.

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is septate.
- Aerial mycelium is also septate; conidiophores are medium long and at the end of diversifying.
- Multiplies with arthroconidia that are sequentially separated from hyphae.

SIGNIFICANCE:

That mould is known as “red bread mould”, sometimes it appears on hazelnuts, beans and meat products, and on storage fruits (apples, strawberries, raspberries). *Chrysonilia* grows very quickly so that in a few days entire medium in Petri dishes is overgrown and even grow out from the Petri dish. Therefore, the work requires special care (problem of laboratory contamination).

PICTURE:

Magnification of microscope:
1. Substrate mycelium: septate thicker than conidiophore
2. Conidiophore: septate and at the end branched
3. Arthroconidia: unicellular
**Cladosporium**

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is blue black.
- Characteristic are large, low, compact, velvety colonies, that have olive green colour.

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is septate.
- Conidiophores are branched, look-like tree, with a delicate structure. Conidiophores sequentially form unicellular conidia (blastic conidiogenesis), which are therefore in appearance similar to budding cells of yeast. Conidiophore is partially or completely decomposed in arthroconidia with buds or scars (from buds). Conidia are often similar in shape (but not in colour) to yeast cells. Conidia are primarily single-cell, at the beginning of development they can also be two-cell.
- Conidia: arthroconidia are unicellular, ellipsoidal shape.

SIGNIFICANCE:

*Cladosporium* occur as saprophytes and pathogens for the crops. Conidia are well adapted to the air scattering, because they are small, dry, highly pigmented and highly resistant to sunlight. As a pathogenic fungus that appeared on fresh fruit and vegetables, typical is spoilage of strawberries, tomatoes. As contaminant they also occur on other foodstuffs. As it grows well at temperatures around 0 °C, they cause spoilage of chilled meat and other foods stored at low temperatures.

PICTURE:

Magnification of microscope:
1. Septate substrate mycelium
2. Septate and branched conidiophore
3. Conidiophore with sequentially formed conidia
4. Single-cell arthroconidia
Curvularia

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is blue-grey to black or brown-black.
- Aerial mycelium is off-white or brown to black. Colonies on CYA are large (diameter of 60 mm or more after incubation at 25 °C for 7 days).

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium: septate, thin.
- Conidiophore: septate, thicker than the substrate mycelium.
- On conidiophore cylindrical, elongated conidia are formed sequentially; Conidia are multicellular and always have transverse septa (4-6 cells). At the beginning conidia are single-cell, but with maturation they become multicellular.

SIGNIFICANCE:

In/on food the most common species is Curvularia lunata, most often on rice, barley and wheat and causing a brown scab. They are also saprophytes.

PICTURE:

Magnification of microscope:
1. Substrate mycelium: septate, thin
2. Conidiophore: septate, strong
3. Conidia: multicellular, an elongate and transversely septate
MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is strongly coloured from yellow-red to violet pigments and they are excreted into the medium, which therefore coloured.
- Aerial mycelium is filamentary, cotton wool, white to pale pink, red, purple or brown. The colonies are large (40-70 mm, CYA, incubation at 25 °C for 7 days) and often outgrow the whole medium.

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is septate. Older hyphae are thicker, younger are thinner and brighter.
- Conidiophores are aseptate, very short, individually or in tufts.
- Conidiophores have phialide which can form two different kinds of conidia:
  - macro-conidia: multicellular in shape like banana, with at least 3 septa
  - micro-conidia: most of the species that form micro-conidia have single-cell conidia that are ellipsoidal and in chains or groups
- Chlamydoconidia: for some species in *Fusarium* are characteristic also chlamydoconidia in substrate mycelium.

SIGNIFICANCE:

*Fusarium* moulds are known plant pathogens. As spoilage microorganisms they cause decay, they are found mainly in fruits and vegetables and cereal products. They form different mycotoxins, which can provoke nausea and intoxications.

PICTURE:

Magnification of microscope:
1. Septate substrate mycelium, older hyphae are
2. Thicker, younger are thinner and brighter
3. Short conidiophores with phialide
4. Multicellular macro-conidia
5. Single-cell micro-conidia
**Geotrichum**

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is a cream, vanilla colour.
- Aerial mycelium is white, cream-colored and extremely low with a compact structure. The colonies have a diameter of 20-45 mm (CYA, incubation at 25 ° C for 7 days).

MICROMORPHOLOGICAL PROPERTIES:

- Substrata and aerial mycelium are septate and interweaves.
- *Geotrichum* multiplies by forming arthroconidia. These are the cylindrical spores which may arise in such a way that part of the mycelium is separated and when mycelium is ripped it is separated into individual arthroconidia.

SIGNIFICANCE:

From *Geotrichum* genus the most important species found in foods is *G. candidum*. It is pathogenic mould of citrus (especially lemon and grapefruit) and causes sour rot. As spoilage occur also on tomato, dried peppers, carrots, cucumbers, onions and beans. It can be as a contaminant present in the processing line (canned, frozen food). The problem is also in in the manufacture of soft, fresh cheeses.

PICTURE:
Magnification of microscope:
1. Thin and septate mycelium
2. Arthroconidia of cylindrical shape
**Scopulariopsis**

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is yellow to light brown.
- Aerial mycelium differs in color from white to light brown; it is never green or blue. Colonies are larger or smaller (40 - 50 mm or 15 to 30 mm on MEA, incubation at 25 °C for 7 days), low and thinner structures at the edges.

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is septate.
- Simple or branched conidiophores, those are also septate.
- Conidiophores have at the end conidiogenic cells – annelids that form conidia. Conidiogenic cells may be individually or on metula similarly as at *Penicillium* (phialide extrude conidia, annelids detached conidia and thus anelide is "extends").
- Conidia are single cell, pear-like shape with a truncated a blunt end, which is best seen after release conidia from anelide. Conidia are large (5 – 8 µm), colourless to brown in colour, often with rough, wrinkled surface.

SIGNIFICANCE:

Mould of *Scopulariopsis* genus are saprophytes and are often found in manure, the decaying, rotting vegetation and in soil. In foods is *Scopulariopsis brevicaulis* namely in rice, rice flour, milk powder, cheese and the butter. For man is pathogenic fungus.

PICTURE:
Magnification of microscope:
1. Septate substrate mycelium
2. Septate conidiophore
3. Anelide
4. Single-cell conidia
**Penicillium**

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is coloured yellow, ocher and brown.
- Aerial mycelium is low, cotton wool, velvet structure. At first is white colour, and then it turns from white, to grey or to green or to blue green. Conidia are blue, green and white or grey.

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is septate, thicker and darker than the air mycelium.
- Conidiophores grow individually or in pairs from basal cell in substrate mycelium. Conidiophores are septate, thinner and more transparent than substrate mycelium.
- Conidiophore is at the end cleaved in the symmetric or asymmetric branching form named metula.
- On metulae are phialides and they forming conidia.
- Conidia are in chains (up to 50), are oval shape and white or green colour.

SIGNIFICANCE:

*Penicillium* moulds are the most important spoilage microorganisms according to the great different foods that they can contaminate. They can also grow at low temperatures of refrigeration and at low $a_w$. They are generally present on cereals and cereals products and on different foods at low temperatures. Many species of *Penicillium* can form mycotoxins. Some species are industrially important as they have proteolytic and lipolitic activity (i.e. in cheese making) or at production of antibiotic agents in pharmacy.

PICTURE:
Magnification of microscope:
1. Substrate mycelium: septate, thicker and darker than the air mycelium
2. Conidiophore: which might be cleaved at the and has metula
3. Phialide: on metulae
4. Round conidia: on phialides in chains up to 50
**Phoma**

**MACROMORPHOLOGICAL PROPERTIES:**

- The mycelium is usually pigmented from grey, brown to green and contains pycnidia. Pycnidia often give black-brown appearance to colonies. Some colonies have a distinctive red shade (*Phoma macrostoma*).

**MICROMORPHOLOGICAL PROPERTIES:**

- *Phoma* moulds are forming pycnidia. Pycnidium is a flask-shaped cavity from the surface of the inner walls of which spores are produced. Pycnidium has one or more gaps - ostiole.
- Moulds produce pycnidiospore – conidia in pycnidium.
- Conidia are mainly unicellular, ellipsoidal to cylindrical shape. They may be coloured or non-coloured.
- Some species form also chlamydospores singly, in chains or in small groups (dichtiochlamydospore at *Phoma glomerata* – look very similar as conidia at *Alternaria*).

**SIGNIFICANCE:**

In *Phoma* genus are more than 200 species. These moulds are very widespread in the soil, often are saprophytes on different plants, some species are plants and human pathogens.

**PICTURE:**
Magnification of microscope:
1. Pycnidium
2. Unicellular conidia
**Trichothecium**

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is orange.
- Aerial mycelium is low, the structure of cotton wool and orange pink colour. Colonies on CYA have a diameter of 50-70 mm (incubation for 7 days at 25 °C).

MICROMORPHOLOGICAL PROPERTIES:

- Substrate and aerial mycelium are septate and very thin.
- Conidiophore is a straight-chain, at the end become rounded and the rounded portion form a conidia that are a "zig-zag" short chains.
- Conidia are two-cell and oval. Development: first conidiophore thickens; cell wall is formed and then unicellular conidia. Then septa is formed and conidium become two-cell. Under the microscope we can see the blank, thick one and two-cell conidia in a typical formation of V-shape.

SIGNIFICANCE:

*Trichothecium* has only one species of *T. roseum*, which is saprophyte and sometimes pathogen for plants and humans. On foods such as wheat, flour, corn, rice, apples, grapes occurs as saprophytes. In general, as spoilage moulds on foods are not often present.

PICTURE:

Magnification of microscope:
1. Substrate and aerial mycelium
2. Conidia: mostly two-cell and oval
**Trichoderma**

MACROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is colourless.
- Colonies are of low growth, aerial mycelium is initially colourless, later white-green colour. Colonies grow very rapidly (diameter of 4-9 cm in 5 days on potato agar). Aerial mycelium when maturing forms fluffy blue green fields with "knots" conidia.

MICROMORPHOLOGICAL PROPERTIES:

- Substrate mycelium is septate.
- Conidiophores, which are also septate, grow singly or in groups from substrate mycelium. Conidiophores form irregular pyramid branched in tree formation.
- At the end of conidiophore are phialide, which are at one side oval to the other tapered. Phialide are variable length and individually or, more rarely in groups of 2 to 4 and come from conidiophore in different angles.
- On phialide are round (sometimes oval) conidia with rough surface (Trichoderma viride) or smooth surface (Trichoderma harzianum).

SIGNIFICANCE:

*Trichoderma* form small conidia, which rapidly spread through the air and contaminate air - so care is needed when working with this mold in the laboratory. Many moulds of *Trichoderma* form chitinase and cellulases, which can eventually completely destroy other microbial cultures. *Trichoderma* (particularly *Trichoderma harzianum*) are spoilage of apples and vegetables (peas), grains and because they grow well in the interior are causing rot mainly in citrus (especially *Trichoderma viride*).

PICTURE:
Magnification of microscope:
1. Substrate mycelium
2. Conidiophore, which is septate and form tree-like structures
3. Phialide of different length on conidiophore
4. Unicellular conidia
IDENTIFICATION OF YEAStS TAKEN INTO ACCOUNT FOR THEIR IDENTIFICATION

Main characteristics that are determined at the identification of yeasts are microscopic (shape and size of cells, ways of reproduction), cultural (growth on a solid and in liquid medium) and biochemical characteristics (oxidative and fermentative consumption of carbon compounds, utilization of nitrogen, the formation of starch, formation of different enzymes) and ecological properties (relation to oxygen, relation to pH, relation to temperature, relation to osmotic pressure, growth in presence of antibiotics).

MICROSCOPIC CHARACTERISTICS

Shape and size of cells:
The yeast cells can have different shapes (round, oval or egg-shaped, ellipsoidal, oblong or irregular shape) and size (average size: with round cells: the diameter of 4-6 µm in oval cells: 4 -6 µm width, length 6-10 µm, the elongated cells, the width of 4-6 µm, length 12-16 µm).

Ways of vegetative (asexual) reproduction:

BUDDING
When a cell reaches maturity for budding, on a particular location of the cell walls small bulge rapidly increasing and cells buds. After the splitting of nucleus bud dispense from maternal cells and thus new cell of yeast is forming. At the point where the new cell dispenses remains on the surface of the mother cell and the bud cells round scar. At this point, the cell can no longer germinate. In optimal conditions, this cycle lasts about two hours.

For certain types of yeast formation of pseudomycelium may occur in such a way that the newly formed bud does not dispense from the mother cell and...
begins itself to germinate. Sequential repetition of this process yield chains of linked yeast cells, which are called pseudomycelium.

For some of the yeast is characterized budding only at one end (polar budding), some may budding at the two opposite ends (bipolar budding) and when yeasts are capable of budding at any point of the cell is called multipolar budding.

**BINARY FISSION**
Some types of yeast reproduce like bacteria with binary fission. When the cell reaches maturity, the nucleus divides, these two new nuclei move away and in the middle of the cell a double transverse wall is formed, and then the cells are separated. When rapidly growing yeast cells is divided, without being separated from each other a true mycelium is created.

**FRAGMENTATION**
Those yeasts which temporarily form pseudomycelium or mycelium can reproduce with fragmentation, that is, the formation of arthrospores or oidia. (Trichosporon).

**BLASTOSPORES**
A blastospore is an asexual fungal spore produced by budding from mycelium or pseudomycelium.

**CHLAMIDOSPORES**
They are formed only in certain yeasts such as thickening of the mycelium.

**Ways of generative (sexual) reproduction:**

**FORMATION OF ASCUS WITH ASCOSPORES**
Increasing the number of yeast with sexual reproduction is often slower than growth with asexual reproduction.

Number of ascospores in ascus is characteristic for each species of yeast, for example, only 1-2 ascospores or 4 ascospores or 8 ascospores or other number of ascospores in ascus.

For each type of askosporogenic yeast are typical number of ascospore and the shape of ascospores which can be globe to ovoid, elliptical, kidney or peas similar or moon-like, like a hat or helmet, characteristic is also the surface of ascospores which can be smooth, knobbly or warty.

**CULTURAL CHARACTERISTICS**
The form of growth in a liquid medium: growth on the bottom, or on top or form of turbidity in the test tube.
Growth on solid medium: characteristic colour, shape, cross-section, edge and surface of colonies on selected medium.

**BIOCHEMICAL CHARACTERISTICS**

**Oxidative and fermentative utilization of carbon compounds:**
Yeast can use sugars and certain organic compounds as a carbon source. The sugars may be used by fermentation - it is in the processes of anaerobic and on oxidative way. Individual sugars may be used only by certain types of yeast and therefore help us in their identification.

**Utilization of nitrogen:**
Ammonium salts (such as ammonium sulphate) are the preferred source of yeast nitrogen - nitrogen from the ammonium sulphate can be used by all yeasts. Only a small number of yeast can exploit nitrogen from the nitrate. Therefore, we use this ability of yeast to assimilate nitrates for identification.

**Formation of starch or starch-like substances:**
This test helps to identify yeasts in *Cryptococcus* genus, as it is for certain species characteristic to form extracellular polysaccharides.

**Formation of urease:**
Some yeasts have the enzyme urease other do not have this enzyme.

**ECOLOGICAL PROPERTIES**

**Relation to oxygen:**
This test indicates whether the yeasts are aerobic, anaerobic, or microaerophilic and this can be determined in parallel with the cultivation of a culture in a liquid medium.

**Relation to pH:**
Pure culture is inoculated in culture media with different pH values and after incubation figure out where the best growth is (such as pH 2.5, 3.5, 4.5).

**Relation to temperature:**
Pure culture is incubated at different temperatures, such as 20° C, 24° C in 37° C and after incubation figure out where the best growth is.

**Relation to osmotic pressure:**
Spoilage yeasts can grow at very high sugar concentration (60%) and they are determined as osmophilic yeasts.

**Growth in presence of cycloheximide:**
Some yeast can grow in the presence of 0.01% cycloheximide (an antibiotic which acts in many fungi, it is an inhibitor of protein synthesis at the ribosomes of eukaryotes).
INSTRUCTIONS FOR PERFORMING TESTS FOR IDENTIFICATION OF YEASTS

Tests with labels 2, 3, 5, 7, 8, 9 is carried out with pure cultures grown on solid culture media, the tests with labels 1, 4, 6 is carried out with suspensions of pure cultures in physiological saline. Pure cultures are labelled with I to VIII.

1. GROWTH OF YEASTS IN LIQUID MEDIA

1 ml of pure culture is inoculated in liquid medium, glucose yeast peptone (GKP), suspension is mixed and after 7-day incubation at 28 °C on a shaker determine form of growth: sediment, turbidity, surface ring, islets on the surface, raised edges.

Then the suspension is mixed and native microscopis slide is prepared by using 3 inoculationg loops of suspension. The cell shape (round, oval, ellipsoid, oblong, lemon, raindrop) of cells and the way of vegetative reproduction (budding: unipolar, bipolar, multipolar; binary fission; or a combination of both) are determined.

2. GROWTH OF YEASTS ON SOLID MEDIA

Pure culture is inoculated onto solid medium (GKP, glucose yeast peptone agar) with an inoculation loop (three dots). After 7-day incubation at 28 °C shape of colonies, colour of colonies, surface of colony (flat, smooth, smooth with a hill in the middle, smooth with craters in the middle, rough with the crater in the middle, smooth mycelium growth medium, smooth, curly, convex) and the edge of the colony (smooth, wavy , teeth, on pinnatifid, curled) are determined.

3. SPORULATION

Pure culture is inoculated on solid medium for sporulation (McClyr acetate agar) with an inoculating loop ("zig-zag"). After prolonged incubation of 2-6 weeks at 28 °C prepare stained microscopic slide. We use modification of Kinyoun method (acid-fast stain). On a slide put in a small drop of water and spread culture in it by inoculating loop, dry the smear on the air next to burner, fix the smear and stain it:

- carbol fuchsin 5 min
- pour off the stain
BASIC PRINCIPLES OF IDENTIFICATION OF MOULDS, YEASTS AND BACTERIA

- washed it with 70% ethanol
- distain it with a solution of HCl + ethanol
- washed it with water
- methylene blue 1 min
- washed it with water
- dry it with a paper towel

Add a drop of immersion oil and observe the slide under 1000x magnification. Vegetative cells and ascus are blue, ascospores are red. The number of ascospores in ascus, their shape and size (spheroidal and smooth, kidney, smooth, rough spheroidal, rough, oblong, lance, conifer, helmet-like) are also important for identification of yeast.

4. FORMATION OF PSEUDO-MYCELIUM OR MYCELIUM
Two inoculation loop of yeast suspension is inoculated on potato agar medium on a slide, which is in a petri dish (preparation of microculture). The medium with the culture is covered with coverslip. A piece of cotton wool soaked in sterile water is added in Petri dish. After a 7- to 14-day incubation at 28 °C microculture is observed under the microscope and formation of pseudo or true mycelium is determined.

5. ASSIMILATION OF NITROGEN COMPOUNDS
Pure culture is inoculated ("zig-zag") on two solid culture media containing a carbon base, one media has potassium nitrate and the second has ammonium sulfate. After 7 days of incubation at 28 °C is a positive reaction the growth of colonies on the medium.

6. GLUCOSE FERMENTATION
Pure culture (1 ml) is inoculated in a liquid culture medium containing 0.5% yeast extract and 50 mM glucose, and Durham tube. After 7-day incubation at 28 °C a positive reaction is presence of gas in Durham tube.

7. FORMATION OF EXTRACELLULAR STARCH-LIKE COMPOUNDS
Pure culture is inoculated on solid medium ("zig-zag"). After 7-day incubation at 28 °C we determine formation of starch-like compounds by addition of lugol's iodine solution and positive reaction is blue-violet colour.

8. UREASE TEST
In liquid medium - Christensen inoculate pure culture with an inoculation loop and mix the suspension. Incubation of media is at 37° C, and each 30 min (up to 2 days) determine color change and a positive reaction is purple colour.

9. GROWTH IN PRESENCE OF 0.01% CYKLOHEXIMIDE
On solid medium with D-glucose and with cycloheximide (0.01%) inoculate pure culture with an inoculation loop "zig-zag". After 7 days of incubation at 28 °C is a positive reaction growth of colonies on the medium.
MAIN FEATURES OF SELECTED YEAST CULTURES

*Candida*
These yeasts form in liquid medium sediment, on solid media colonies have crater and are white with a rough surface and a serrated edge. Cells have oval or cylindrical shape and multipolar budding. They have no sexual reproduction. Pseudomycelium is formed. They do not grow on the medium with KNO₃ and not on the medium with cycloheximide. Yeasts ferment glucose, does not form extracellular starch-like compounds and they have urease.

*Debaryomyces*
These yeasts form cream-white colonies on a solid medium. Yeasts have a round or oval cells which multipolar budding. Some species form a simple pseudomycelium. They form 1-2 ascospores in ascus. Ascospores are round or oval and have a warty surface. Carbohydrates are not fermented or very poor. They do not grow on the medium with nitrate and lack of urease. Some species can grow on a medium containing cycloheximide

*Hansensenucla*
These yeasts form in liquid medium sediment, colonies on solid media are cream white colour with a smooth surface and craters and wavy edges. The cells are round or oval with multipolar budding. They form 1-4 ascospores that look like hat with a smooth surface. They do not form a mycelium or pseudomycelium. They ferment glucose, utilise nitrates, do not form starch, no urease and do not grow on the medium with cycloheximide.

*Hanseniaspora*
These yeasts form in liquid medium sediment, colonies on solid media are cream-white, flat, with shiny surface and smooth edge. Yeast cells are apiculate, oval and elongated shapes and bipolar budding. They form 1-2 ascospores similar to the hat or helmet with rough surface. Pseudomycelium is not formed. They do not grow on the medium with KNO₃, ferment glucose, do not form extracellular starch-like substance and have no urease. They grow in the presence of cycloheximide.

*Pichia*
On solid media colonies are cream white colour with a smooth shiny surface and smooth edge. Yeast cells are oval and multipolar budding. Pseudomycelium does not form or form a simple pseudomycelium. They are typically 4 ascospores in askus. They ferment glucose, some strains utilise nitrates. They do not form a starch-like compound and have no urease, they do not grow in the presence of cycloheximide.
Rhodotorula
In liquid media they form form sediment and turbidity; on a solid culture medium the colonies are orange red with a smooth surface and an edge, and have crater. The cells are oval with polar budding. Ascospores are not formed. Some species form pseudomycelium. The colonies grow on the medium with KNO$_3$ and do not grow on the medium with cycloheximide. Yeast can ferment glucose and other sugars, and do not form extracellular starch-like compounds, but they have the enzyme urease.

Saccharomyces
In liquid media they form form sediment and turbidity and ring; on a solid culture medium the colonies are cream-white with a smooth surface, a crater and have smooth edge of colony. The cells are round or cylindrical shape and multipolar budding. They form 1-4 or more ellipsoidal smooth ascospores. Pseudomycelium is not formed, but individual hyphae may occur. They do not grow on the medium with KNO$_3$ and do not grow in the presence of cycloheximide. They ferment glucose, they do not form a extracellular starch-like compounds, but they have urease.

Schizosaccharomyces
In liquid media they form form sediment; on solid medium colonies are cream-white, the surface is smooth and has a central raised center, the edge is smooth. Cells are cylindrical shape and are divided. Yeasts form 1-4 smooth ascospore. They do not form a pseudomycelium, do not grow on the medium with KNO$_3$ and not on the medium with cycloheximide and do not form a extracellular starch-like substance. They ferment glucose and have urease.

Zygosaccharomyces
On solid medium colonies are cream-white. They reproduce by budding, and with ascospores - up to four ascospores are in askus. The cells are oval, and do not form a mycelium or pseudomycelium. They ferment glucose, do not form starch-like substance and have no urease. They do not grow on the medium with cycloheximide and not on the medium with KNO$_3$. 
**YEAST I.**

Describe properties and write them in table!

<table>
<thead>
<tr>
<th>PROPERTY</th>
<th>DESCRIPTION OF RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Growth in liquid GKP medium:</td>
<td></td>
</tr>
<tr>
<td>• Form of growth</td>
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<tr>
<td>• Form of cell</td>
<td></td>
</tr>
<tr>
<td>• Vegetative reproduction</td>
<td></td>
</tr>
<tr>
<td>2. Growth on GKP agar:</td>
<td></td>
</tr>
<tr>
<td>• Colour of colony</td>
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<tr>
<td>• Surface of colony</td>
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<tr>
<td>• Edge of colony</td>
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<tr>
<td>3. Formation of ascospores</td>
<td></td>
</tr>
<tr>
<td>4. Formation of pseudomycelium</td>
<td></td>
</tr>
<tr>
<td>5. Assimilation of nitrogen:</td>
<td></td>
</tr>
<tr>
<td>• (NH(_4))_2SO(_4)</td>
<td></td>
</tr>
<tr>
<td>• KNO(_3)</td>
<td></td>
</tr>
<tr>
<td>6. Fermentation of glucose</td>
<td></td>
</tr>
<tr>
<td>7. Formation of starch</td>
<td></td>
</tr>
<tr>
<td>8. Urease</td>
<td></td>
</tr>
<tr>
<td>9. Medium with s cycloheximide (0.01 %)</td>
<td></td>
</tr>
</tbody>
</table>

Draw pictures of slides and describe the main features!

Native slide: ___________________________

Coloured slide: ___________________________

Yeast strain I. is

...........................................................................................................................................................................
## YEAST II.

Describe properties and write them in table!

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Draw pictures of slides and describe the main features!
Native slide: Coloured slide:

Yeast strain II. is………………………………………………………………..
YEAST III.

Describe properties and write them in table!

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<td>5. Assimilation of nitrogen:</td>
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<td>8. Urease</td>
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<td>9. Medium with s cycloheximide (0.01 %)</td>
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Draw pictures of slides and describe the main features!
Native slide: Coloured slide:

Yeast strain III. is …………………………………………………………………..
Describe properties and write them in table!

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Draw pictures of slides and describe the main features!
Native slide: 
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Yeast strain IV is………………………………………………………………………………
YEAST V.

Describe properties and write them in table!

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Draw pictures of slides and describe the main features!
Native slide: Coloured slide:

Yeast strain V. is .................................................................
Describe properties and write them in table!

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Draw pictures of slides and describe the main features!
Native slide: 
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Yeast strain VI. is .................................................................
YEAST VII.

Describe properties and write them in table!

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Draw pictures of slides and describe the main features!

Native slide:  
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Yeast strain VII. is .................................................................
Describe properties and write them in table!

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Draw pictures of slides and describe the main features!
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Yeast strain VIII. is ..............................................................
IDENTIFICATION OF BACTERIA

Identification schemes are not classification schemes. The identification scheme for a group of microorganisms can be made only after the group of microorganisms are classified, which means that, according to certain properties different from the other groups. In general, the properties of organisms selected for the identification scheme, may be easily determinable. Likewise, these properties should be as little or as possible or as it is strictly necessary for identification.

MAIN CHARACTERISTICS OF BACTERIA TAKEN INTO ACCOUNT FOR THEIR IDENTIFICATION

Each organism should be first classified, that means placed in a particular group or classification scheme. According to the classification then identification scheme for practical use is made.

The main characteristics of bacteria for their classification and identification are morphological, physiological, metabolic and serological properties, phagetyping, ecological characteristics, genetic analysis and molecular properties.

MORPHOLOGICAL PROPERTIES

Micromorphological properties:

Shape of cells: cocci (often round, sometimes oval or ellipsoid), bacilli or rod-shaped bacteria, a curved shape (vibrio, spirile, spirochetes).

Formation of cells: Cocci can be individually (monocoque) or may be combined in pairs (diplococcus), chains (streptococci), in form of a cluster (staphylococci), in form of tile which has a thickness of only one coccus (Micrococcaceae) or in the form of cubes (Sarcina). Rod-shaped bacteria can be individually ((mono) bacillus) or grouped in the pairs (diplobacili) or chain (streptobicili).
The size of bacteria: The size of the bacterial cells is different, and is measured by a micrometer, 1µm = 10^{-6}m (i.e. rod shaped bacteria of *Escherichia coli* are 1 x 3 µm), while eukaryotic cells are larger (diameter may be greater than 200 µm).

Gram staining: cells of Gram-positive bacteria are stained blue-violet, cells of Gram-negative bacteria are pink-red.

Capsule: For many bacterial cells wall overlaps another more or less thick layer, which is called a capsule. Structure of capsules varies (polysaccharides, polypeptides).

Spore-formation: Some bacteria produce endospores, which may be smaller than the diameter of the cell: a terminal, or central position, sub-terminal or greater than the diameter of the cells.

The number and location of flagella: is for species or strain characteristic property.

Macromorphological properties:

Shape of colony (dotted (2r <1mm), round (2r > 1 mm), irregular), edge of the colony (smooth, serrated), section of colony (low, raised, torus, convex), colour of colony (most bacteria form a colourless to white, crème colonies, some bacteria produce pigments, for example, *Staphylococcus aureus* form a carotenoid and colonies are yellow), surface of colony (smooth, matte, shiny, slimy, granulated, with concentric circles radially serrated).

**PHYSIOLOGICAL AND METABOLIC CHARACTERISTICS**

Physiological and metabolic properties of bacteria are very important because they are a direct reflection of the nature and activity of bacterial enzymes and transport proteins. Because the proteins are conditioned by the genetic composition, analyses of these characteristics allow indirect comparison of bacterial genomes. Among the most important physiological and metabolic characteristics are: use of different sources of carbon and nitrogen, fermentation products, mechanisms of energy conversion, motility, osmotic tolerance, attitude towards oxygen (strict anaerobes, facultative anaerobes, strict anaerobes and microaerophilic), formation of secondary metabolites and the effects of metabolic inhibitors and antibiotics.

**SEROLOGICAL FEATURES AND PHAGE-TYPING**

Microorganisms act as antigens in a host to cause the production of antibodies. Antisera are commercially available solution of antibodies that can be used in the identification of bacteria, as the antibody - antigens are very specific. The antibodies may be species-specific or specific for the respective strains.

Phage-typing means determining the phage to which the sensitive bacteria and is used to distinguish strains within a single species.
ECOLOGICAL CHARACTERISTICS
Examples of important taxonomic characteristics are life cycle, nature of the symbiotic relationship, ability of the bacteria that cause disease (pathogenicity), host properties such as pH, temperature, oxygen, ..., and the optimum temperature, optimum pH.

GENETIC ANALYSIS
Among the most important genetic characteristics of the bacteria is study of exchange of genetic material in transformation and conjugation, and a study of plasmids.

MOLECULAR PROPERTIES
In the classification, the study of proteins and nucleic acids is an important part of the research. The amino acid sequences of the proteins directly reflect the sequences of mRNA molecules, and thus the structure of the genes responsible for their synthesis. Comparison of proteins of different bacteria is taxonomically very important feature. The proteins can be compared directly to compare the amino acid sequences of proteins of the same function. Since they are quite time-consuming analysis, often they make use of rapid methods, such as electrophoretic mobility of proteins and reaction antibody - antigen. Increasingly important area of studies is study of nucleic acids (RNA, DNA) as their independent analysis of phenotypic traits comparison of bacterial genomes it is possible in several ways:
• determining the mol% G + C of the DNA in the bacterial mol% G + C ranging between 25% and 80%, but in spite of such a wide area, however, this% for a given type of constant and characterized.
• various hybridization techniques
• the most accurate data is obtained by sequencing of (part of) DNA

Numerical taxonomy
Numerical taxonomy means a quantitative approach or way of data processing and, by definition, taxonomic grouping units into groups based on equivalent properties with numerical methods. This means that the data on the properties of organisms organize for computer processing and to perform numerical analysis. The classification is based on the general similarity of organisms. For precise and reliable classification is used from 50 to some 100 characteristics of an organism. Calculated similarity between the isolates used for their classification into different taxonomic levels. If the similarity is 90% or more to isolate qualify for the same group.

Standardized methods
The results of the various tests are dependent on the size of the inoculum, incubation temperature, time of incubation, the composition of medium, relationship between surface area and volume of the medium, and many other factors. Accordingly, all the conditions need to be standardized, which is implementing a very challenging and not always feasible. In a lot of help are
commercial systems (eg API). It is necessary to also compare the results of the investigated strain with the results given by strains whose identity is determined (reference strains).

**Definition of positive and negative results**
Test results should be reproducible and clearly defined as positive and negative results. Since such tests is not ideal, it is necessary to precisely define what is positive and what is negative result and only then a particular test may be useful in the identification scheme.

**Pure culture**
Pure culture is, with few exceptions, always necessary to carry out identification tests. Recent identification techniques using genetic probes or polymerase chain reaction (PCR), may also be implemented in mixed microbial populations.

**General procedure of identification**
1. Isolation of pure microbial cultures
2. Continuation of the work from broad categories to more specific organism
3. Use all the information that is most narrowed scope of possible organisms
4. Use the minimum number of tests
5. Comparison of isolates with the reference strains

**GRAM-NEGATIVE BACTERIA**

According to Bergey Manual bacteria are divided into 4 sections, three sections are bacteria and one archaea (I. Gram-negative bacteria with a cell wall, II. Gram-positive bacteria with a cell wall, III. Bacteria without cell walls, IV. Archaea). Each section is further divided into classes and those in orders, families, genera and species.

In Bergey Manual are Gram-negative bacteria classified into 7 groups, and of these are important for the food industry in particular, the following:

**GROUP 2: AEROBIC / MICROAEROPHILIC, MOTILE, HELICAL OR VIBRION GRAM-NEGATIVE BACTERIA**
They are gram-negative cells or vibion helical shape (less than one turn), motile. They have a respiratory metabolism and require oxygen as a final electron acceptor. They are aerobes or microaerophilic. They are found in ground water, sea water, plants, digestive tract of animals and humans. Some species are pathogenic to human. 
Genera: *Campylobacter*

**GROUP 4: GRAMNEGATIVNE AEROBNE / MIKROAEROFILNE PALČKE IN KOKI**
They are gram-negative rods or cocci with the respiratory type of metabolism and in need of oxygen as a final electron acceptor. They are located in the
GROUP 5: FAKULTATIVNO ANAEROBNE GRAMNEGATIVNE PALČKE
They are gram-negative rods, which can grow under aerobic conditions and
have a respiratory type of metabolism or under anaerobic conditions to the
fermentation type metabolism. They can be wild or in association with humans,
animals or plants. Some species are pathogenic.
SUBGROUP 1: family Enterobacteriaceae
Genera: Citrobacter, Enterobacter, Erwinia, Escherichia, Hafnia, Klebsiella, Kluyvera,
Proteus, Salmonella, Serratia, Shigella, Yersinia
SUBGROUP 2: family Vibrionaceae
Genera: Aeromonas, Vibrio

GRAM-POSITIVE BACTERIA
In Bergey Manual are Gram-positive bacteria without actinomycetes divided
into 6 groups, and of these the most important for the food industry following:

GROUP 17: GRAM-POSITIVE COCCI
They are Gram-positive cocci and mesophilic bacteria. They are aerobes,
facultative anaerobes or strict anaerobes.
Genera: Enterococcus, Micrococcus, Pediococcus, Sarcina, Staphylococcus, Streptococcus

GROUP 18: SPOROGENIC GRAM-POSITIVE RODS AND COCCI
They are characterized by the formation of endospores that are resistant to
heat (70° - 80° C, 10 min). Most of them are motile bacteria, they are aerobes,
facultative anaerobes, or anaerobes or microaerophili.
Genera: Bacillus, Clostridium

GROUP 19: NON-SPOREFORMING GRAM-POSITIVE RODS
They are sporeforming, Gram-positive bacteria, non-pigmented, mesophilic.
Some species are pathogenic.
Genera: Brochothrix, Lactobacillus, Listeria

GROUP 20: NON-SPOREFORMING GRAM-POSITIVE RODS
Genera: Acetobacterium, Bifidobacterium

Genera: Acetobacter, Acinetobacter, Alcaligenes, Brucella, Flavobacterium, Legionella,
Moraxella, Neisseria, Pseudomonas, Xantomonas

ground water, sea water, the plants, the digestive tract of animals and humans.
Some species are pathogenic to human.
INSTRUCTIONS FOR PERFORMING TESTS FOR IDENTIFICATION OF BACTERIA

BASIC TESTS

1. GROWTH ON SOLID MEDIUM

For this test use a solid non-selective medium (such as nutrient agar, NA) and a pure culture is inoculated by inoculating loop (isolation). After 24-hour incubation at 37°C following properties are determined:
* colony morphology: shape, edge and colour
* morphology of the cells: microscopic smear according to Gram staining and observation of: cell shape, cell formation and reaction to the Gram stain.

2. SPORE-FORMING

Spore-forming bacteria are determined from test 1 by making a microscopic smear stained according to Schaeffer-Fulton.

3. CATALASE

Mix one colony from the test 1 in three drops of H₂O₂ (3%) on a slide and appearance of bubbles of oxygen means that bacterium has catalase. Catalase is an enzyme formed by some of actively growing aerobic bacteria. Strict anaerobes lack the ability to use oxygen for respiration and therefore do not have catalase.

Positive result:

\[ 2\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} \text{O}_2 + 2\text{H}_2\text{O} \]  
(bubbles)

4. GROWTH IN SEMI-SOLID MEDIUM
Use a semi-solid medium for motility of bacteria with 0.3% agar and inoculate pure culture with an inoculating needle. After 24-hour incubation at 37°C determine growth of bacteria:

- uniform growth – non-motile bacteria
- tree-like growth – motile bacteria
- growth throughout medium - motile bacteria

5. RELATION TO OXIGEN

Use the solid medium which is prior to use dissolved and cooled to 45 °C. The pure culture (0.1 ml) is inoculated in a culture medium and the suspension is gently stirred. After 24-hour incubation at 37°C relation to oxygen is determined:

- growth only on the bottom of tube - anaerobic bacteria
- growth throughout medium - facultative anaerobic bacteria
- growth on the surface of medium - aerobic bacteria
- growth just below the surface of medium - microaerophilic bacteria

6. SUGARS FERMENTATION

Use a liquid culture medium containing a peptone, a sugar, pH indicator and Durham tube. The medium is labelled 6a when glucose and brom cresol purple indicator are in medium and 6.b when lactose and indicator brom thymol blue are added. Inoculate medium with 1 ml of a pure culture, mix the suspension gently and after 24-hour incubation at 37°C observe the formation of acid and the occurrence of gas in Durham tube.

Positive result:

sugar $\rightarrow$ acid (pH is lowered, yellow colour) and gas in Durham tube (or no gas in Durham tube)

7. PROTEOLYSIS

Use a semi-solid medium (meat-peptone broth, MPB) with the addition of gelatine (120 g/l) and pure culture is inoculating with an inoculating needle. After 24-hour incubation at 37°C we need to cool medium in refrigerator because the gel at temperatures above 20°C is fluid. Then we observe liquefying of gelatine that mean that have investigated bacteria have extracellular proteolytic enzymes gelatinase.

Positive result:

$\text{gelatine} \xrightarrow{\text{gelatinase}} \text{polypeptides (chains of amino acids)}$

$\text{polypeptides} \xrightarrow{\text{gelatinase}} \text{individual amino acids (fluidized medium)}$
ADDITIONAL TESTS
According to the results of basic tests an additional test(s) is selected to identify the investigated bacterial culture.

1. HAEMOLYSIS
Use solid medium - blood agar (KA), which contains the blood and inoculate culture with inoculating loop (solution). Blood agar is an enrichment and differential medium on which the different types of haemolysis is determined. After 24-hour incubation at 37°C observe:
- formation of green zone around the colonies means α-haemolysins partially degrade haemoglobin
- formation of clear zones around colonies means that β-haemolysins completely decompose haemoglobin
- Growth of colonies without zone means that culture does not have haemolysins.

2. LIPOLYSIS
Inoculate the culture (in "line") on solid medium Tributyrin agar which as an individual carbon source contains Tributyrin. After 24-hour incubation at 37°C was determine lipolytic bacteria with the growth of colonies which have around colony a transparent zone.

3. FORMATION OF INDOLE
Investigated culture is inoculated in peptone water containing tryptophan (amino acid with the indole ring). After 24-hour incubation at 37°C observe formation of indole with the addition of Ehrlich's reagent and Kovac's reagent. In the case of positive reactions of indole, it is seen as red ring.

Positive result:
\[
\text{tryptophan} \xrightarrow{\text{tryptophanase}} \text{NH}_3 + \text{pyruvic acid} + \text{indole}
\]
\[
\text{indole} + \text{Ehrlich's reagent} + \text{Kovac's reagent} \rightarrow \text{red coloured complex}
\]

4. METHYL RED
Culture is inoculated in a liquid medium with glucose (MR-VP) and mixed. After 2-5 days of incubation at 35°C we can find out a way of converting glucose. Most of Enterobacteriaceae in 24 hours converts glucose to pyruvic acid. Some species of Enterobacteriaceae in following 2-4 days convert pyruvic acid into a variety of acids (lactic, acetic, formic acid), which is detected by the addition of methyl red indicator, when medium turns red. At a pH of 6.0 is methyl red indicator of yellow colour.

Positive result:
\[
\text{glucose} \rightarrow \text{pyruvic acid (after 24 h of incubation at 35°C)}
\]
\[
\text{pyruvic acid} \rightarrow \text{variety of acids, pH < 4.2}
\]
5. VOGES PROSKAUER

From the culture medium in test 4 prove formation of acetoin. After incubation, decant 2.5 ml of the suspension into a new test tube and add solution of α-naphthol (6 drops) and a solution of KOH (2 drops). Mix and if in 15 minutes formation of a red color means presence of acetoin and positive result of VP.

Degradation of glucose in certain Enterobacteriaceae species can take place over pyruvate to neutral products such as acetoin. Acetoin is after addition of KOH and under the influence of oxygen from the air is converted into diacetyl. This forms a red complex in the presence of α-naphthol

Positive result:

\[
\begin{align*}
glucose & \rightarrow pyruvic acid \\
pyruvic acid & \rightarrow acetoin \\
actein + \alpha-naftol + KOH & \rightarrow \text{red colour of medium}
\end{align*}
\]

6. CITRATE

Inoculate pure culture on solid culture medium with citrate as a sole carbon source. After 24-48 hours incubation at 37°C observe growth of bacteria and the change in colour of the medium. In medium is added an indicator bromothymol blue (green at pH 6.9, blue at pH 7.6). Bacteria having an enzyme citrase grow on the medium, exploiting citrate and thereby alter pH of the medium, which can be seen by changing the colour of the medium from green to blue.

Positive result:

\[
\begin{align*}
citrate & \rightarrow \text{oxalyl-acetic acid + acetic acid} \\
oxalyl-acetic acid & \rightarrow \text{pyruvic acid + CO}_2 \\
\text{CO}_2 + \text{Na}^+ & \rightarrow \text{Na}_2\text{CO}_3 (\text{pH is increased})
\end{align*}
\]

Tests formation of indole, methyl red, Voges Proskauer, and citrate are mainly used for the identification of Enterobacteriaceae and are commonly referred to as IMViC.

7. GROWTH IN MEDIUM CONTAINING POTASSIUM CYANIDE

Inoculate pure culture in liquid medium with KCN and mix content. After 24-48-hour incubation at 37°C observe growth of bacteria such as appearance of turbidity of the medium.
GRAM STAINING

Gram-positive bacteria have relatively simple cell wall, consisting of:

- cytoplasmic membrane
- peptidoglycan (or murein) up to 25 layers

Gram-negative bacteria have multi-layer cell wall, consisting of:

- cytoplasmic membrane
- periplasm with a thin layer of peptidoglycan
- lipopolysaccharides and proteins

In Gram-positive bacteria cell wall is thin and ethanol cause dehydration - peptidoglycan pores are reduced and the color complexes of crystal violet-lugol remain in the cell wall. In Gram-negative bacteria ethanol is going through a peptide layer and a thin layer of peptidoglycan and dissolve the colour complexes of crystal violet-lugol. Gram staining is differential staining procedure which separates most bacteria into two groups on the basis of cell wall composition:

- Gram-positive bacteria (thick layer of peptidoglycan-90% of cell wall)-stains purple,
- Gram-negative bacteria (thin layer of peptidoglycan-10% of cell wall and high lipid content) –stains red/pink

Put a drop of water on microscopic slide, take a small sample of colony and spread it to 1-2 cm². Let it air dry and then fix it and stain according to procedure described below.

Steps:

<table>
<thead>
<tr>
<th>Staining</th>
<th>Colour of Gr+:</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chryystal violet</td>
<td>blue/purple</td>
<td>blue/purple</td>
</tr>
<tr>
<td>2 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washing with water</td>
<td>blue/purple</td>
<td>blue/purple</td>
</tr>
<tr>
<td>Lugole</td>
<td>purple</td>
<td>purple</td>
</tr>
<tr>
<td>2 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washing with ethanol (96%)</td>
<td>purple</td>
<td>colorless</td>
</tr>
<tr>
<td>Washing with water</td>
<td>purple</td>
<td>colorless</td>
</tr>
<tr>
<td>Safranin</td>
<td>purple</td>
<td>red</td>
</tr>
<tr>
<td>30 s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washing with water</td>
<td>purple</td>
<td>red</td>
</tr>
</tbody>
</table>

Microscopic smear is dried with a paper towel, then immersion oil is added and microscoped (R = 1000x).
STAINING OF BACTERIAL ENDOSPORES

For microscopic separation of bacterial endospores and vegetative cells we use staining according to Schaeffer-Fulton. Primary dye, malachite green, is heated at fixed smear so that dye can penetrate through relatively impermeable envelopes of endospores. Malachite green also binds to vegetative cells, but in next step the rinsing water wash it out from cells and washed vegetative cells are colourless. In order to increase the contrast of the cell saphranine is added and it binds only to vegetative cells, and therefore they are red coloured.

Put a drop of water on microscopic slide, take a small sample of colony and spread it to 1-2 cm$^2$. Let it air dry and then fix it and stain according to procedure described below.

Steps:
• Smear is covered with a paper towel
• pour a 5% solution of malachite green
• heating for 3 minutes (do not boil or dry it out!!)
• 1 min on room temperature
• Washing with water
• pour a 0.5% safranin solution for 30 s
• Washing with water

Microscopic smear is dried with a paper towel, then immersion oil is added and microscoped (R = 1000x).

MAIN CHARACTERISTICS OF SELECTED BACTERIA

*Campylobacter*

Bacteria of *Campylobacter* are commensal in the intestine of many warm-blooded wild and domestic animals are often found in poultry and cattle. Therefore, raw milk and meat is often the source of epidemic enteritidis. Thermophilic species of *Campylobacter jejuni* and *Campylobacter coli* are pathogenic for humans. The disease usually begins after the ingestion of food contaminated with a small number of pathogenic bacteria. Signs are diarrhoea, accompanied by nausea and abdominal cramps.

Bacteria of *Campylobacter* are Gram-negative spiral, curved and moving rods. They are microaerophilic bacteria with the respiratory type metabolism. They grow in an atmosphere of 3% to 15% O$_2$ and 3% to 5% of CO$_2$. They have oxidase and catalase, do not take sugars. Does not hydrolyse gelatine, MR and VP were negative and have no lipase. Thermophilic species of *Campylobacter jejuni* and *Campylobacter coli*, *Campylobacter lari* and *Campylobacter upsaliensis* grow at 42°C and not at 25 °C. Species of *Campylobacter jejuni* and *Campylobacter coli* are differentiated with hydrolysis of hippurate: *C. jejuni*: positive, *C. coli*: negative.
**Basic Principles of Identification of Moulds, Yeasts and Bacteria**

**Pseudomonas**

*Pseudomonas* bacteria are widespread in nature (in soil, water and air, on plants), are often isolated from fresh foods (vegetables, meat, poultry). They are the most important group of food-spoilage bacteria causing great range of different spoilage of foods at low temperatures because many species and strains are psychrotrophs. Major species are *P. aeruginosa*, *P. fragi* and *P. fluorescens*. *P. aeruginosa* can be responsible for the poisoning of people with food when food contamination is large $N > 10^6$ cfu/g (mL)).

Bacteria of *Pseudomonas* are Gram-negative rods without special formation (single cell). They are aerobic bacteria with a strictly respiratory type of metabolism and in need oxygen as a final electron acceptor. Having one or more polar flag - are motile bacteria having catalase enzyme and proteolytic and lipolytic enzymes. They use glucose, but no lactose.

**Escherichia**

Bacteria of *Escherichia* are normally in the gut of humans and warm-blooded animals. The most important species is *Escherichia coli* known also as an indicator of food hygiene. *E. coli* is transmitted directly from animal to man, from person to person and through contaminated food. Most serotypes are non-pathogenic, while some serotypes can be very dangerous and pathogenic for humans (for example, O55, O111, and O128). Enteropathogenic strains cause severe diarrhea and vomiting, and infections are very dangerous for infants and children. Enteropathogenic strains can also form enterotoxins.

*E. coli* are Gram-negative rods grouped in pairs or irregular groups. They are motile, facultative anaerobic bacteria, which grow best at 37° C (enteropathogenic strains at 42- 44 °C). They have respiratory and fermentation type of metabolism. They use glucose and lactose and form acids and gas. They have catalase, but lack of proteolytic enzymes. Test results of IMViC are + + - -.

**Salmonella**

Bacteria of *Salmonella* are widespread; their main natural environment is gastrointestinal tract of humans and animals. The hosts are excreted them into the environment with faeces, and bacteria can survive for several years, and from there spread to ground, water, sewage. Because of inadequate sanitation these bacteria could also come into the food. *Salmonella* was isolated from various foods especially of animal origin (poultry, pork, beef, eggs, raw milk). It is known over 2,000 serotypes of *Salmonella*, and most of them are pathogenic to humans and animals. *Salmonella* in humans cause various illnesses and are responsible for many foodborne diseases. Salmonellosis is a common term for infection of humans and animals with salmonela. *Salmonella* serotypes S. Typhi and S. Paratyphi cause very dangerous diseases with the highest mortality rate.
Salmonella are Gram-negative, rod-shaped bacteria. They are motile and facultative anaerobic bacteria. Glucose transforms to acid, gas usually do not form, lactose is not used. They have no oxidase, but have catalase. They do not grow in the medium with KCN. They do not form indole and acetoin from glucose and can grow on citrate as the sole carbon source. They do not hydrolyse gelatine.

**Proteus**

Bacteria of *Proteus* are found in soil, water, waste water, human and animal faeces and rotting waste. As spoilage they were isolated from a variety of vegetable and meat products and eggs. They cause mesophilic spoilage of foods stored at room temperatures.

Bacteria of *Proteus* are Gram-negative rods which are individually, in pairs or short chains. They have more flagella and are motile. They are facultative anaerobic bacteria with the respiratory and fermentative metabolism. They utilize glucose and form acid and gas, but not lactose. They have catalase and proteolytic enzymes, and grow in a medium with KCN. Genus *Proteus* contains different species and some are described in the table.

<table>
<thead>
<tr>
<th>Test / Species</th>
<th>P. mirabilis</th>
<th>P. myxofaciens</th>
<th>P. penneri</th>
<th>P. vulgaris</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Methyl red</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>d</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citrate</td>
<td>d</td>
<td>+</td>
<td>-</td>
<td>(-)</td>
</tr>
<tr>
<td>Formation of H₂S</td>
<td>+</td>
<td>-</td>
<td>d</td>
<td>+</td>
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<tr>
<td>Hydrolysis of gelatine</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>+</td>
</tr>
</tbody>
</table>

Legend: + … more than 90% of the strains is positive, - … more than 90% of the strains is negative, d … 11-89 % strains is positive

**Enterococcus**

The primary source of bacteria of genus *Enterococcus* is the gastrointestinal tract (gut) of humans and animals, often are also found on plants, soil and insects. They may serve as an indicator of food hygiene and water (indicators of faecal contamination) and they were also isolated from a variety of foods such as cheese, meat products, milk. The food is mostly contaminated with *Enterococcus pyogenes* and *Enterococcus faealis*. *Enterococcus pyogenes* causes respiratory diseases. *Enterococcus faealis* is a contaminant in several hams, sausages and cream. It causes mild form gastroenteritis, incubation is 2-18 hours and is manifested as nausea, diarrhea and vomiting.

Bacteria of the genus *Enterococcus* are Gram-positive cocci, which are in pairs or short chains. They are facultative anaerobic bacteria, and non-motile. *Enterococcus faealis* use lactose, are catalase negative and form β-hemolysis on a blood agar.
**Staphylococcus**

Bacteria of *Staphylococcus* are on skin and mucous membranes of humans (nasal cavity, hands, face, eyes, throat, and intestinal tract) and warm-blooded animals. From here they can come in the air, dust particles, on clothing and other places, which may be a source of contamination of food by staphylococci. Most of species form coagulase, nuclease and/or enterotoxins and among them are *Staph. aureus, Staph. epidermidis, Staph. intermedius* and *Staph. haemolyticus*. The most studied species is *Staph. aureus*, as they are known, some strains to cause gastroenteritis. In general, staphylococci can all contaminate foods of animal origin and those foods that come in direct contact with humans, if not subsequently included technological process which could destroy them.

*Staphylococcus* are Gram-positive cocci that are individually, in pairs and in the irregular groups. They are facultative anaerobic bacteria, and non-motile. They can form carotenoid pigments. They have catalase and form acids from glucose and lactose. They are proteolytic and they have β - haemolysin.

**Bacillus**

Bacteria of *Bacillus* are widespread in nature (earth, air, water, plants) and are often isolated from various foodstuffs of animal and plant origin (for example, cereal products, potato products, vegetable soup). The genus *Bacillus* contains pathogenic species *B. anthracis* (anthrax) and *B. cereus* at which some strains are pathogens. Consuming foods of animal origin and a variety of cooked meat dishes, which have been contaminated with *B. cereus* was often cause of poisoning (abdominal pain and diarrhea after 8 to 16 hours)). These foods have greater concentration of *B. cereus* ($10^5$-$10^8$ cfu/g). Another form of poisoning (nausea, vomiting, after 1 to 5 hours) is associated with the consumption of cooked rice which was after cooking, and stored for a longer time at room temperature. *B. cereus* strains thus forming at least two types of toxins (heat-unstable toxin (destroyed in 30 min at 56 °C) and heat-stable toxin (not destroyed after 90 min heating at 126 °C)). For bacteria in genus *Bacillus* is characteristic that they are spore-forming bacteria, they can be psychrotroph, mesotroph or thermophilic, they can be acidophilic, and some species are even halotolerant.

*B. cereus* are Gram-positive bacteria with central or sub-terminal endospore. The cells have the shape of long rods, which are individual, in pairs or short chains. They are facultative anaerobic and aerobic bacteria, and are motile. They have catalase and proteolytic enzymes. They use glucose, but not lactose, MR and VP are positive and characterized by α-hemolysis on blood agar.

**Clostridium**

Bacteria of *Clostridium* are widespread nature (earth, water, air, plants), they are also present in human and animal intestinal tract. Most species are anaerobic,
even though some types can proliferate in the presence of oxygen (for example, *Cl. perfringens*). The genus *Clostridium* contains several species of human pathogens such as *Cl. butulinum* and *Cl. perfringens* (forming different exotoxins). Infection with *Cl. butulinum* is very severe (12 to 72 hours to appear nausea, vomiting, headache, a feeling of dryness of skin, mouth, throat, and later constipation, drop in body temperature, paralysis of muscles, double vision, and ultimately failure of the respiratory system) and the duration of the 1 to 10 days, mortality rate is between 30 and 65%. Consuming foods heavily contaminated with *Cl. perfringens* shows after 6-24 hours signs such as diarrhea, abdominal pain and nausea, usually after 24 hours the symptoms disappear. *Clostridium* contains mesotrophic, psychrotrophs and thermophilic species. Strains are also important as spoilage bacteria in ready-to-eat foods and canned goods.

*Clostridium* are Gram-positive, sporogenic rods, which are often found in pairs or short chains. Most species is an obligatory anaerobic and motile. They do not have catalase.

**Listeria**

*Listeria* includes bacteria that are ubiquitous in nature. They relatively long survive in adverse conditions, they grow in a wide temperature range from 0.4° C to 45 ° C, at a pH of 4.4 to 9.6 and *a*_ w of 0.92. The result is a relatively common occurrence of these bacteria in foodstuffs, such as: raw milk, cheese, meat, poultry, vegetables. For human *L. monocytogenes* is pathogenic and the most common source of infection is consumption of contaminated food. Listeriosis is an infection with several different forms of which is meningitis, it is dangerous for pregnant women which can lead to miscarriage or other complications. Epidemiological data indicate that mortality is the highest among bacterial infections with contaminated food.

*Listeria* are Gram-positive, non-spore forming rods. They have catalase, they are motile between 20 ° C and 25 ° C and facultative anaerobic bacteria. For *L. monocytogenes*, is typical that they form β-haemolysis on blood agar that utilizes rhamnose and does not use xylose, and a positive test CAMP. For *L. innocua*, is typical that they do not have haemolysis on blood agar, utilize xylose and not rhamnose, and has a negative test CAMP.
**BACTERIUM I.**

Describe properties and write them in table!

<table>
<thead>
<tr>
<th>PROPERTY</th>
<th>DESCRIPTION OF RESULTS</th>
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<tbody>
<tr>
<td>1. MORPHOLOGY</td>
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<td>• Gr+ / Gr-</td>
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<td>2. ENDOSPORE</td>
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<td>3. CATALASE</td>
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<td>4. MOTILITY</td>
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<td>5. ATTITUDE TO O₂</td>
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<td>6. FERMENTATION</td>
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<td>• Glucose</td>
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<td>• Lactose</td>
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<td>7. PROTEOLISIS</td>
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<td>ADDITIONAL TEST:</td>
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</table>

Draw pictures of slides and describe the main features!

Slide stained according to Gram: Slide stained according to Schaeffer-Fulton:

Bacterium I. is .............................................................
# BACTERIUM II.

Describe properties and write them in table!

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</table>

Draw pictures of slides and describe the main features!

Slide stained according to Gram:  [Image]
Slide stained according to Schaeffer-Fulton:  [Image]

Bacterium II. is  ..............................................................................................................
BACTERIUM III.

Describe properties and write them in table!

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<thead>
<tr>
<th>PROPERTY</th>
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Draw pictures of slides and describe the main features!

Slide stained according to Gram:                Slide stained according to Schaeffer-Fulton:

Bacterium III. is ..............................................................
BACTERIUM IV.

Describe properties and write them in table!

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ADDITIONAL TEST:

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Draw pictures of slides and describe the main features!

Slide stained according to Gram: Slide stained according to Schaeffer-Fulton:

Bacterium IV. is..........................................................................................................................
BACTERIUM V.

Describe properties and write them in table!

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<td>• Lactose</td>
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<td>7. PROTEOLISIS</td>
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</table>

**ADDITIONAL TEST:**

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Draw pictures of slides and describe the main features!

Slide stained according to Gram:  
Slide stained according to Schaeffer-Fulton:

---

Bacterium V. is......................................................................................................................
BACTERIUM VI.

Describe properties and write them in table!

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Draw pictures of slides and describe the main features!

Slide stained according to Gram: Slide stained according to Schaeffer-Fulton:

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Bacterium VI. is........................................................................................................................................
**BACTERIUM VII.**

Describe properties and write them in table!

<table>
<thead>
<tr>
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Draw pictures of slides and describe the main features!

Slide stained according to Gram:    Slide stained according to Schaeffer-Fulton:

![Slide stained according to Gram](image1)

![Slide stained according to Schaeffer-Fulton](image2)

Bacterium VII. is

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**BACTERIUM VIII.**

Describe properties and write them in table!

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Draw pictures of slides and describe the main features!

Slide stained according to Gram: Slide stained according to Schaeffer-Fulton:

Bacterium VIII. is...........................................................................................................
TEXT-IMAGE KEYS FOR IDENTIFICATION OF MOULDS

I. KEY

1.A. In the colonies dominate budding cells, sometimes pseudomycelium, or very rarely vegetative mycelium .............................................. YEASTS

1.B. Colonies have a lot of vegetative mycelium, conidia or other asexual spores are formed in or on specialised cells ................................................................. 2

2.A. Mycelium has normally no septa or very few, asexual spores are mostly formed in sporangium .................................................. ZYGOMYCETES

2.B. Mycelium is usually with septa, sexual or asexual spores are not formed in sporangia .................................................................................................................. 3

3.A. Asexual spores are formed on or in specialised cells—conidiogenic cells or from conidiphores ......................................................... DEUTEROMYCETES

3.B. Sexual spores are formed in askuses or basidia................................................. 4

4.A. Sexual spores are formed in askuses.................................................. ASCOMYCETES

4.B. Sexual spores are formed in basidia.................................................. BASIDIOMYCETES
II. KEY: ZYGOMYCETES

1.A. Sporangiospores are formed in cylindrical merosporangium that has no columella ........................................................... *Syncephalastrum*

1.B. Sporangiospores are formed in rounded or pear-like sporangium that has columella .........................................................

2.A. Sporangia and sporangiophores are dark colour, sporangiophores are mostly unbranched and are often in groups, spores are often with more rugged surface .......................................................... *Rhizopus*

2.B. Sporangia and sporangiophores are not pigmented or are ali pa so brightly colored, sporangiophores are often branched, spores have smooth surface .....................

3.A. Sporangia have shape that look like pear and have an average diameter of 10 to 40 µm ................................................................. *Absidia*

3.B. Sporangia are round and have an average diameter greater than 40 µm ............................................................................. *Mucor*

III. KEY: DEUTEROMYCETES

1.A. Conidia are formed in picnidia ................................................................... *Phoma*

1.B. Conidia are not formed in picnidia, but on hyphae, conidiophores or on/in other conidiogenic cells ..................................................

2.A. Conidia se tvorijo v posebnih konidiogenih celicah: phialide,anelide, ali zaobljeni deli Conidiophorea, v verižicah ali v obliki glavic ............ *3*

2.B. Conidia se ne tvorijo v posebnih konidiogenih celicah .................................. *14*

3.A. Conidia are in chains ............................................................................. *4*

3.B. Conidia are in groups ............................................................................. *10*
4.A. Conidia are mostly double-cells, colonies are orange or pink orange ................................................................. *Trichothecium*

4.B. Conidia are always single-cell, colonies are of different colours ............................................................................................................ 5

5.A. Colonies are small and red-brown. Conidia are formed on fertile hypha by splitting off in groups of four ......................................................... *Wallemia*

5.B. Colonies are normally growing, conidia are not formed by splitting from fertile hypha ........................................................................................................ 6

6.A. Conidiophore has apex ............................................................................................................................... *Aspergillus*

6.B. Conidiophore has no apex .......................................................................................................................... 7

7.A. Conidiogenic cells are anelide, conidia have typically on one side a severed tip ........................................................................................................ *Scopulariopsis*

7.B. Conidiogenic cells are phialide, conidia have no a severed tip ................................................................................................................................. 8

8.A. Colonies are dark grey to black, phialide are egg-like shape, conidia are blackish ............................................................................................... *Memnoniella*

8.B. Colonies are not dark colours, phialide are shape of flat bottle ................................................................................................................................. 9

9.A. Colonies are yellow to brown, phialide have long neck ......................... *Paecilomyces*

9.B. Colonies are often greenish (sometimes with white dots), phialide have short neck ..................................................................................................... *Penicillium*

10.A. Phialide are long, they have shape of awl, no polyphialide .................... *Acremonium*

10.B. Phialide are in shape of flat bottle, with or without polyphialide ....................................................................................................................... 11
11.A. Colonies are usually greenish................................................................. Trichoderma
11.B. Colonies are off-white, yellow, purple, pink, brown
or blackish........................................................................................................ 12

12.A. Colonies are off-white, yellow, pink, purple, sometimes
greenish, multicellular conidia have typical shape of banana ...................... Fusarium
12.B. Colonies are black, sometimes pink, conidia have no septa................. 13

13.A. Individual phialide are on individual, unexpressive
conidiophores................................................................................................. Phialophora
13.B. Phialide grow in groups on long conidiophores
that have septa or have no septa.................................................................. Stachbotrys

14.A. Very fast growing colonies (in a few days outgrow medium in Petri dish),
pinkish orange with rare and tenuous wool structure.................................. Chrysonillia
14.B. Colonies are not rosy orange ................................................................. 15

15.A. Conidia are unicellular........................................................................... 16
15.B. Conidia are multicellular........................................................................ 18

16.A. Conidiophores are with many septa, asymmetrically branched and
at ends form ellipsoidal conidia in tufts......................................................... Botrytis
16.B. Conidiophorei are branched with a delicate structure......................... 17

17.A. Conidia are colorless to cream white color, they have a smoothsurface, and a more or
less cylindrical shape arthroconidia .............................................................. Geotrichum
17.B. On branched parts of conidiophore are formed arthroconidia that look
like budding yeasts cells.................................................................................. Cladosporium

18.A. Conidia mostly have just lateral septa.................................................... Culvularia
18.B. Mature conidia have transverse and longitudinal septa....................... Alternaria
IV. KEY: ASCOMYCETES

1.A. Ascocarps are clearly separated on air hyphae................................................. Monascus
1.B. Ascocarps are not separated on air hyphae .......................................................... 2

2.A. Colonies grow extremely slowly, they grow only on media for xerophiles,
asci have 2 ascospores in shape of a crescent moon.................................................. Xeromyces
2.B. Colonies grow faster, asci have usually 8 ascospores ........................................ 3

3.A. Ascocarps (peritecia) are covered with hyphae................................................ Chaetomium
3.B. Ascocarps (cleistotecai) are not covered with hyphae,
ascuses are oval or round shape .............................................................................. 4

4.A. Ascocarps are with no special wall................................................................. Byssochlamys
4.B. Ascocarps are with special envelope............................................................... 5

V. PHOTOS OF NATIVE MICROSCOPIC SLIDES

Native microscopic slides of moulds observed under a light microscope at 400x magnification. Other enlargements are listed at the photos.
Fig. 1: *Acremonium*

Fig. 2: *Acremonium*

Fig. 3: *Alternaria*

Fig. 4: *Alternaria*

Fig. 5: *Aspergillus*

Fig. 6: *Aspergillus*

Fig. 7: *Botrytis*

Fig. 8: *Chaetomium* (100x)

Fig. 9: *Chaetomium* (600x)
Fig. 10: *Chrysonilia*

Fig. 11: *Cladosporium*

Fig. 12: *Curvularia*

Fig. 13: *Fusarium*

Fig. 14: *Fusarium* (600x)

Fig. 15: *Geotrichum*

Fig. 16: *Mucor* (100x)

Fig. 17: *Mucor*

Fig. 18: *Penicillium*
Fig. 19: *Penicillium*

Fig. 20: *Phoma* (100x)

Fig. 21: *Phoma*

Fig. 22: *Rhizopus* (100x)

Fig. 23: *Rhizopus* (100x)

Fig. 24: *Scopulariopsis*

Fig. 25: *Scopulariopsis* (600x)

Fig. 26: *Trichoderma*

Fig. 27: *Trichothecium*
LITERATURE


http://www.botany.utoronto.ca/ResearchLabs/MallochLab/Malloch/Moulds/Moulds.html (8.7.2008)