UNIVERSITY OF TARTU
INSTITUTE OF MOLECULAR AND CELL BIOLOGY

CHAIR OF GENETICS

EXPERIMENTAL MICROBIOLOGY

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PREFACE

The current material „Experimental microbiology" is set up in such a way that it would follow and enhance the lecture course. Here are presented main practical works from classical microbiology and also new contemporary research methods for microbiology. The study material gives the basics on inoculating, incubating, isolating, observing and identifying microbes. Laid out in differently themed sections are the instructions for practical works which are preceded by theory to explain the principles of those works. These explanations might be missing from the lecture materials, because the current material is tightly coupled to the practical works. After each theoretical section a questionnaire is presented – answers to which are needed to understand the material inside practical works section. To avoid over-inflating the size of the "Experimental microbiology" minimal amount of explanatory figures, equations and diagrams are used, as these can also be found in the textbooks about of general microbiology. On the other hand, it is taken into account that the participant in practicum could use the material to protocol the work and make relevant notes. The current material helps to save time that would be spent to explain the theory before each practicum, therefore helping to succeed better in the experimental tests.

The material is laid out in 9 themed section with practical works all covering 60 academic hours and is orientated mainly for biology, gene- and environmental-technology students to carry out microbiology practicum. The study material is composed by E. Heinaru (introduction and themes I, II, III, IV, V, VII, IX) and E. Naanuri (theme VI, co-author E. Heinaru, theme VIII, co-authors E. Heinaru, Signe Viggor, Merike Jõesaar).

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Authors

INTRODUCTION TO MICROBIOLOGY PRACTICUM

A. THE MICROBIOLOGY LAB WORK PROCEDURE AND SAFETY INSTRUCTIONS

In addition to safety practices related to dealing with regular toxic and caustic materials one has to take into account the specifics of microbiological work.

1. Regardless of the pathogenicity of microbial material the **rules of personal hygiene must be followed**. Work is done wearing protective clothing and separate footwear must be worn. Eating, drinking and smoking in the lab is forbidden. Avoid raising hands to mouth, eyes and hair. **After working in the lab hands must be washed!**
2. **Follow the procedures related to your tools.** The working surface must be kept clean of un-necessary items. The inoculation-needle, -loop and -spatula have to be **sterilized** in flame also **after the use.** On no occasion should one attempt mouth pipetting. All tools that have had contact with microbes will be placed into appropriate container. Picks, cotton, paper-strips and other such disposables should also be placed into appropriate containers. The staining of specimens will be carried out over staining container that will be used for no other purpose. All disposable contaminated material will go to special containers to be autoclaved. The glassware that has had contact with microbial cultures will be collected and cleansed.
3. When working with **UV-radiation** eyes must be protected by goggles and skin by protective clothing and gloves.
4. **Take care when using immersion oil!** In case of eye contact flush with running water at least for 15 minutes. On contact with skin wash with water and soap.
5. **First aid in case of microbial skin contact.** The affected area must be disinfected with 70% ethanol or 3% chloramine and afterwards washed with soap and water. In case of any skin injuries on hands gloves must be used for all work.

B. THE BASICS OF MICROSCOPY

The present practicum uses three types of microscopes: bright-field, phase contrast and fluorescence. For this reason we will expound on the working principles of these three. The microscope is a main tool for a microbiologist and one is generally a pretty expensive piece of equipment. From it follows, that as in practicum one microscope will be used by lot of different people, then all of them should know the separate pieces of a given microscope, its working principle and the principles of its sustainable use.

1. THE MICROSCOPES

1.1. Bright-field microscope

To inspect specimens in microbiology practicum usually a bright-field microscope is used – where by strongly illuminating the specimen the microbes will appear darker as caused by different absorption of light. The rays emanating from the light source will travel through condenser, the lenses of which will bring them together on the specimen. The rays that have traveled through the specimen will reach the objective lenses. The image produced by objective is furthermore magnified by ocular lens (eyepiece).

The microscope consist of the microscope's body to which the following standard components are attached: binocular tubes housing eyepieces, the objective revolver on which objectives are housed, the specimen stage (possibly a mechanical stage with added controls to finely move (slide) the specimen preparation) and a condenser equipped with iris diaphragm.

The binocular tubes are affixed to the microscopes body with a fastening screw. They contain a slider ring for regulating optical power (known as a diopter ring) and are also equipped with the possibility to regulate the distance between the eyepieces. The mechanical stage will have knobs to regulate the sliding of the specimen preparation both in x- and y-axis. The two knobs on the side of the microscope (usually housed inside each other) allow for coarse and fine adjustment of focus. On the foot of the microscope stands a lamp that will have an on-off switch and a voltage regulator to control the light intensity.

The microscope is equipped with three systems of lenses – oculars lenses (eyepieces), objectives and the condensers. In the simplified schema of a microscope there is only two convex lenses: one with a short focal distance, (focus being the point in optical axis where the rays previously parallel with optical axis that travel through the lens will gather) called objective lens which will give a magnified real and inverted image A_1B_1 from an object AB and another with a large focal distance, called ocular lens that will create magnified upright virtual image A_2B_2. For the linear magnifications of objective and ocular lenses s_1 and s_2 $A_1B_1/AB=s_1$ and $A_2B_2/A_1B_1=s_2$. When we multiply the appropriate sides of these two equations with each-other we will get $A_2B_2/AB=s_1s_2$ **meaning that the magnification of the microscope is the product of the magnifications of objective and ocular lenses.**

The magnification of the microscope can be regulated by using eyepieces and objectives with different focal distance. For maximal magnification objectives with short focal distance are used. When the objective is said to be in focus it means that the specimen lays in the focal point F of objective. This distance is also known as working distance which should remain constant.

The larger the magnification of the objective the shorter the working distance. For objectives with magnifications 10x, 40x and 100x the working distance are respectively 10.5, 0.56 and 0.13 mm. Focal distances are proportional to the diameter of the lens.

The third system of lenses is the condenser, which is situated under the specimen stage and has the function of collecting the rays of light on the specimen. This functionality is achieved by using a concave mirror or by combining opposite-facing plano-convex lenses in series. The iris diaphragm of condenser controls the diameter of the light beam. By opening-closing the diaphragm we can change the intensity of the light flux on the specimen. In case of the microscopes with no light intensity regulator on the lamp it remains the only way to regulate the intensity of the light. Like with all the lenses the condenser also has a focal point. For this reason it is important to focus the condenser. Under the stage a button for sliding the condenser up and down is situated. Many microscopes are built in such a way that in the uppermost position of the condenser the F is on the specimen. The position of condenser will determine how well the diameter of the light cone will match the diameter of the objective lenses.

Trying to use microscope on objects that are close to the wave-length of light the bending of light know as diffraction around these objects becomes apparent. The created diffraction rings cover each other and will not allow us to differentiate objects that are small enough and close to each-other. From this follows that the magnification of the microscope is limited by its resolution meaning the closest distance d between two points that one can differentiate by using given microscope. The Abbé equation illustrates this ability to give a detailed image of the specimen:

$$\boldsymbol{d = \frac{0.61\times\lambda}{n\times sin\frac{\alpha}{2}}} \qquad \textbf{\textit{or}} \qquad \boldsymbol{d = \frac{1.22\times\lambda}{(NA_{objective}+NA_{condenser})}},$$

where λ is the wave-length of the light used (avg. 0.55 μm), α – the angle between the outermost light rays that still reach the objective, **n** – the refraction index of the environment between the specimen and the objective's first lens. The product **n x sinα/2** is called the **numerical aperture (NA)** of the objective and it illustrates the objective's capability to focus light. The larger the NA the smaller the distance between two points that still allows them to be seen separately i.e. the better the resolution. The constant 0.61 (1.22) is used when the condenser's NA is larger or equal to the objective's NA.

According to Abbé's equation it is **possible to increase the resolution of a microscope by using light with a smaller wave-length (Example: a fluorescence microscope using UV-light) or by increasing numerical aperture (NA)**. Inside a homogeneous environment the light will travel in a straight line, but will change its course (refract) when crossing to an environment with a different optical density. The refraction of light follows the rule of reversibility of direction, meaning that when a ray falls (incident ray) onto the boundary (interface) between two different environments in the direction of a refracted ray it will be refracted into the direction of the ray that caused that refracted ray (in the direction of the former incident ray).

To the largest possible incident angle (90°) in air corresponds a refraction angle of 42° in glass. Therefore the rays that in glass have an incident angle larger than 42° do no longer have a refraction angle in air and will be reflected back into original environment (total internal reflection).

Also, some of those rays that would have a smaller incidence angle from air to glass than 90°, will not reach the microscope objective when the traveling in the direction from glass to air. Such "loss of rays" can be avoided by using a more optically dense environment between objective and preparation. In this way an immersion system will raise the brightness of the image. Olympus company uses oil with n=1.52 and an oil-immersion objective (such objectives are marked with black circle) with a NA of 1.25 (engraved on objective) inside their immersion system. In the meantime a 40x dry optical system will have an objective with NA of 0.65. Theoretically NA of an objective in a dry system can be about 0.9. In addition to information about aberration correction, magnification and NA, markings about the length of tube and the thickness of cover glass (0.17 mm) are imprinted or engraved on the objective.

Using Abbé's equation introduced above it is possible to calculate the resolutions of different objectives. For dry system with large magnification objective (40x) the resolution will be about 0.52 μm and for oil-immersion objective (10x) about 0.27 μm. Which means that an observer using a microscope cannot differentiate between two points if they are closer than ~0.2 μm. Human eye has a resolution of ~0.1 mm, which means that we can differentiate a hair (~0.1 mm) from its surroundings.

It must be emphasized that the numerical apertures for dry system objectives are smaller than the aperture of the condenser and when working with those objectives one must close up the condenser's iris diaphragm to a narrower position to lessen the scattering of light and increase the contrast of the image.

Objectives with a large NA are effective only when the NA of condenser is similar or larger. Otherwise the flux of light reaching the objective is too weak which limits the full usage of objectives optical properties.

1.2. Phase contrast microscope

The phase contrast microscope was invented by Frits Zernike in 1935 and his work was recognized with Nobel Prize in 1953. While an ordinary bright-field microscope does not adequately differentiate unstained living cells and their organelles from the surrounding environment due to the little difference in refractive index, a phase contrast microscope allows one to study unstained microbial cells as live specimen preparations. The contrast of the image is produced by the interference between a phase shifted light from preparation and

direct light. The rays of light passing through transparent objects will come out of them as direct or, depending on the different optical densities of organelles, refracted in different ways. While the first category of rays will not change their amplitude or phase the latter ones are phase-shifted by a quarter of the wave-length. Zernike's phase contrast microscope has a special phase-ring in front of the condenser to guarantee that after passing the condenser the light will refract in parallel rays, and a phase-plate with phase-ring after the objective, which will shift the direct light by quarter of the wave but will allow the already phase-shifted refracted light to pass without changes. If the direct and refracted light now are in the same phase then we will get brighter image of an object (due to adding up of the amplitudes of the same phased waves). If the rays of light are in opposite phases (out of phase by ½ of wave-length) their amplitudes sum to null and we get a dark image of object. The result of all that is getting an image where the **differences in phase are transformed to differences of contrast observable by eye.**

1.3. Fluorescence microscope

This kind of microscope is characterized by its large field of use. The difference with bright-field microscope is that it is equipped with a mercury-vapor lamp to generate UV-light and instead of Abbé's bright-field condenser a dark-field condenser is used. In addition different filter combinations are used to select specific wave-lengths of UV-light to produce excitation in different fluorescent dyes known as fluorochromes and transmitting the generated light to the observer. The emitted light has a longer wave-length than the light producing the excitation. Some materials have the property of photo-luminescence i.e. they emit the light with a different color than the one that produced excitation. A short timed luminescence is called fluorescence (where excited molecule reaches the lower energy state in less than 10^{-4} s), luminescence taking a longer time is called phosphorescence.

The infra-red light generated by mercury-vapor lamp do not have any importance to fluorescence and will be caught by a special infra-red filter situated in front of the condenser. The visible and UV light pass through a special „dark" filter which removes longer-wave lengths and only rays that will excite the fluorochromes pass through (green, blue, violet and UV rays). To get better contrast in microscopes field of view a dark-field condenser is used, which by redirecting most of UV rays (the condenser will have larger NA than objective) protects the eyes of the observer. Between the objective and eyepiece also lays a colorless barrier filter to remove the remnants of the excitation rays without having an effect to emitted fluorescent light which has a longer wave-length.

The problem with working with fluorochromes is the "fatigue" of fluorochromes after a longer term UV irradiation.

2. REQUIREMENTS OF MICROSCOPY AND USING THE IMMERSION-OBJECTIVE

- A microscope is a fine instrument. Use it with care. Avoid sudden shocks like concussions.
- Before lifting the microscope make sure that the screw affixing the binocular tubes is screwed in tight. Use two hands to lift the microscope, one holding the body and the other supporting the base.
- The intensity of the microscope's illumination system must be **regulated down to minimum** before turning the lamp on or off. Also regulate the intensity down during breaks in microscopy. Turning off the lamp in such case is not necessary.
- The fine focusing should be done by lowering the specimen stand not raising it.
- The specimen stand should be raised to the objective **only by observing it from the side** of the microscope at the same time.

- When working with a dry system take note of how much the iris diaphragm is opened. When magnification is raised the amount of light passing to objective should also be raised. This can be controlled by the use of iris diaphragm.
- Keep the dry system's objectives clean from immersion oil.
- Immersion oil drop should **only** be placed between the objective and the specimen.
- When using the eyepiece coverings while wearing glasses, fold the coverings down. When not wearing glasses fold them up. This will help to keep away distracting light which could otherwise shine between eye and eyepiece.
- You are not allowed to remove and/or change different sub-components of a microscope.
- After finishing your work clean the immersion-objective from the oil residue, using the prescribed method and rotate the objective with the smallest magnification under the path of light.
- Olympus Corporation allows to clean all its glass products with special lens-cleansing paper. To remove immersion-oil a piece of lens-cleaning paper impregnated with a **mixture of ether (70%) and ethanol (30%)** can be used.
- After finishing the work cover the microscope with the dust-cover.

Owing to the fact that the main objective used in microscopy is a 100x immersion objective we look into the specifics of its use.

- Turn on the lamp and regulate its intensity by using the appropriate button.
- In case of binocular eyepieces regulate the distance between the eyepieces so that viewing through them the fields of view cover each other.
- Turn the objective revolver in a way that the objective with small magnification (10x) would be used. The revolver will lock into the position with a small click.
- Using the clamp present on the specimen stand fix the specimen preparation carefully onto the stand.
- Using the x- and y-axis slider knobs move the specimen into the path of light.
- Regulate the iris diaphragm in a way that only a little light would shine on the objective lens.
- Using the coarse and fine focus adjustment knobs bring the specimen to focus.
- Rotate the objective with the next magnification strength (40x, dry system) into working position and re-focus. In the case of para-focal objectives (such a system of objectives where an object focused by one will be in the focus of all others) only a minimal adjustment by the fine adjustment knob is required. Open up the iris diaphragm some more.
- Move the 40x objective out of working position in such a way that a drop of immersion oil could be placed on top of this area on the specimen which is under inspection. Always use Olympus brand immersion-oil.
- Rotate the immersion-objective (marked with black circle) into the working position. For focusing only use fine adjustment knob. In case of air bubbles in immersion oil move the stand a few times back and forth.
- After finishing the observation regulate the specimen stand down. Rotate the immersion objective away from the working position, so it could be cleaned from the oil in the way described earlier. Remove the specimen from the specimen stand.

3. CALIBRATING THE OCULAR MICROMETER

To measure the sizes or number the microbes using a microscope – its ocular micrometer needs to be calibrated. The latter consists of a small glass disc onto which dimensionless scale from 0 to 100 is engraved. We can see this scale by placing the ocular micrometer inside the eyepiece. The calibration is done separately for specific microscope's specific ocular-

objective system, depending on which one of those is currently used. For this purpose an object micrometer is needed, which resembles a microscopy specimen and onto which a standard scale with a minimum increment of 0.01 mm (10 µm) is engraved. When working with an immersion system a drop of oil has to be placed on the scale. After focusing the scale of object micrometer, its starting position has to be moved to overlap the scale of ocular micrometer (grid on the left side of the field of view). Thereafter one has to count how many increments of ocular micrometer fits exactly into how many increments on object micrometer. For example when 20 increments on ocular micrometer coincides with 3 increments (3 x 0.01 mm) on object micrometer, then 1 increment on ocular micrometer grid is 3 x 0.01/20=0.0015 mm (1.5 µm). Knowing the value of the ocular micrometer's increment, we can determine the size of microbial cells in the specimen when we now place the specimen preparation on the stage instead of the object micrometer. Here it is important to remember that the measuring of microbial size should be made in simple wet mount or hanging drop preparation. The microbial sizes are 10-20% smaller inside dried, fixed and stained specimen preparations as compared to their actual size. On many cases also the background staining is used, in which case the specimen is not fixed. To determine the abundance of microbes the amount of microbes inside a certain surface area depicted on the ocular micrometer's grid is counted.

QUESTIONS

1. Why can't we raise the magnification of a microscope infinitively, while we are observing objects that are close to the wave-length of the visible light by their size?
2. What is the resolution of a microscope?
3. What is numerical aperture and on which does it depend?
4. How can we increase the resolution of a microscope?
5. What is the limit of resolution to a microscope that uses visible light?
6. How is it possible to increase the resolution by using shorter wave-length light?
7. What function does the condenser have? Describe its position during microscopy.
8. When compared to objective, what numerical aperture a condenser has to have?
9. What is the function of iris diaphragm?
10. Compare the openness of an iris diaphragm in dry system to openness of one in an immersion system. Explain.
11. What happens to our field of view and its illumination when we increase the magnification of the objective?
12. What is the working distance of an objective and why do we need to know it?
13. What are the advantages of using an immersion system?
14. Why do we need to increase the amount of illumination when using phase contrast microscope as compared to bright-field microscope?
15. What is the wave-length of fluorescent light compared to exciting light?
16. If the diameter of our field of view is 2 mm then how many bacterial cells with a length (diameter) of 2 µm arranged into a straight line (chain) fit into it?
17. If we can see a chain of 40 microbial cells inside the field of view of an objective with 20x magnification then how many can we see inside the field of view of 100x objective?
18. What is the diameter in µm for a field of view for an objective with 40x magnification when an objective with 20x magnification has a field of view 4 mm in diameter?

I THE PREPARATION, STAINING AND OBSERVATION OF MICROSCOPY SPECIMENS

The linear size of most bacteria fits into the range of 1 to 10 μm, although multi-celled filamentous bacteria might reach to 500 μm in size and the smallest mycoplasma can have linear sizes of 0.1-0.2 μm. Bacteria with extreme sizes have also been found - like *Epulopiscium fishelsonii* (80 x 600 μm) or *Thiomargarita namibiensis*, whose globular cell might have a diameter of 750 μm. While the lengths of bacterial cells can vary depending on the phase of growth the diameter is much more constant. For most bacteria it is about 1 μm, while in the meantime the diameter of a yeast cell is an order of magnitude larger. Bacteria are mainly single-celled organisms. In those cases when after the division the cells remain connected to each other, cellular aggregates form. In this way dual-cells, tetrads, chains, plates, clusters and cube shaped packages can form. Based on morphology bacteria is divided into four main groups – rod-shaped bacteria called bacilli (singular bacillus), sphere-shaped bacteria called cocci (sing. coccus) and thirdly spiral (and curved) bacteria including comma shaped vibrios, S-shaped spirilla and twisted spirochetes among themselves. Fourth there also exist irregular shaped bacteria which can have projections and be budding, filamentous and branched. The strict adherence to certain body shapes inside species and genus shows the adaption of bacteria to certain environmental conditions. The changes to cell shape depend on the growth medium, growth speed and physical parameters of the environment.

A. THE BASICS OF STAINING MICROBES

Microbial cells are almost colorless and because of their high water content do not stand out from the surrounding environment. To discern details, microbial preparations have to be stained. For this a number of different dyes and methods are used. Dyes do not move freely to a living cell – for this reason the cells are killed beforehand. The dye diffuses through the dead cells cytoplasmic membrane and is adsorbed on the negatively charged components of cytoplasm (nucleic acids, teichoic acids, lipopolysaccharide membrane) and is not removed by follow up washing. Usually the dyes are salts where the color is provided by one of the ions. For example the methylene blue which exists as a chloride has the coloring component the positive ion (cation): $MBCl = MB^+ + Cl^-$. These kind of dyes where the coloring ion is positive are called basic or cationic dyes. They stain poorly in the case of low environmental pH, because the negative charge of the cell drops due to bonding with H^+ ions from the environment. Basic dyes are methylene blue, safranin, crystal violet etc.

The other group of dyes are acidic or anionic dyes, which are often Na-, K-, Ca- or NH_4-salts which when ionized will give a negatively charged chromogenic (staining) part. For example the Na-salt of eosin ionizes in following way: Na^+ + eosinate$^-$. In addition to eosin also nigrosin and acid fuchsin and some others are known as acidic dyes. Anionic chromophore does not stain the cell. From this follows the differentiation of microscopic staining to **direct and indirect or negative staining.** In the first case the cell will be stained. In the second case, due to the use of acidic dye, the cell is not stained but the background around the cell will be. The latter staining methods will give a pretty good image of the cell's shape and are used to measure the microbial cells or when microscoping such bacteria that are hard to stain or have very small cross-section (like spirochetes). Often such back-ground staining is used to determine capsules.

Different dyes also differ by their staining strength which depends on the electrical charge of the chromogenic part. For example – the methylene blue stains weakly (staining takes place during 30-60 seconds), crystal violent has medium staining strength (10 s) and carbol fuchsin (basic fuchsin + phenol) stains strongly (5 s). Depending if the staining is

performed by one dye or by combining several it will be called as a **simple or a combined (multiple) staining**. During simple staining a whole cell is evenly stained. Under combined staining there we find **differential staining** which is meant to differentiate different kind of bacteria (gram-positive, gram-negative, acid-fast) and **special staining**, which helps to differentiate specific parts in a microbial cell (endospores, capsule, flagellae, storage materials).

B. WAYS TO STUDY BACTERIAL MORPHOLOGY

Bacterial morphology is studied in two ways – either by studying live preparations or preparations made from killed cells. The most common mistakes in making a specimen preparation are: **taking too much sample material, covering the preparation only partially with dye when staining and letting the dye dry up on preparation.**

1. STUDYING LIVE CELLS

To observe the shape, size and motility the specimen preparations (also called mounts/smears) are made from live cells. To make such preparations glass slides with thickness of 1-1.4 mm are used as the bottom slides of the mount and glass slides with thickness of 0.16-0.17 mm are used as cover slips. To de-grease the slides they are kept in 70% ethanol solution, from which they are picked up using tweezers and before use the ethanol is removed by placing the slide into the flame.

Simple wet mount (smear preparation). Using the loop a small drop of microbial suspension is taken from the broth or a small drop of water is taken after which a small amount of culture from solid medium is mixed into this drop of water to make a weak suspension. After that the drop will be covered with cover slip (using tweezers) in a way that the cover slip is first placed into contact with the drop before lowering it to the microscope slide. This helps to avoid the formation of air bubbles under the cover slip**.** The excess suspension leaking out from the edges of cover slip is dried using filter paper as to avoid „swimming-" or „floating-" preparation. In case we do not use any extra measures to avoid the drying of the smear (like placing vaseline or colorless nail polish under the edges of the cover slip) the slide is ready to be observed immediately.

Live staining using Ficker method. A simple wet mount is stained using indifferent dyes (methylene blue or neutral red) which do not significantly damage the cells. Next to the edge of the covering slip a drop of day is placed which is then sucked under the cover slip by placing a piece of filter paper next to the opposite edge of the cover slip. The staining occurs over a few minutes.

Hanging drop mount. To study cell's motility a „hanging drop" is created. For this a special microscope slide with a depression is used. The edges of the depression are smeared with vaseline. Then the slide is placed upside down onto a larger cover slip on which a drop of microbial culture already rests. Turning this slide now upside down we have a „hanging drop mount" ready to be studied in microscope. In this the drop hangs above the depression in the microscope slide. The real motion of a bacterial cell should not be confused with the Brownian motion which is characterized by small „shaky" movements. This phenomenon is characteristic to colloidal solutions where the particles in the solution „bombard" the colloid particle. A more thorough description of bacterial motility is in the section 2.2.2. of current thematic.

Studying the mount with a phase contrast microscope. As before a simple wet mount is produced. When using the 100x objective of the phase contrast microscope a drop of oil is placed on the top of the cover slide and the preparation is focused in strong light.

After the observations the live preparations are placed into special container where they will be sterilized later.

2. STUDYING PREPARED SMEARS (FIXED SPECIMEN PREPARATIONS)

When live cells are usually observed using dry system objectives then killed microbial cells are studied stained using oil immersion system. Before staining the bacterial cells are killed - fixed. With this a better adhesion of cells to a microscope slide is secured to avoid the washing away of the cells during multiple staining and washing cycles and also a better diffusion of dye to cells is guaranteed. The easiest fixing is heat-fixing. For this a thin smear is made out of microbial suspension which will be **air dried** and fixed inside the flame by drawing the microscope slide through the flame with the side covered with cells facing upwards. The slide should be hot but not burning hot when touching it with the back of your hand. Because the microbes deform in the heat other ways of fixing them are also used. Chemical fixing changes the morphology of the cells less but is more cumbersome and time-consuming. The fixing-solution is placed on the slide in drops or the preparation slide is immersed into the fixing-solution. 96% ethanol (10-15 min), pure methanol (3-5 min), 40% formalin (few seconds), 5% phosphomolybdic acid water solution (5 min) are used as fixing solutions. Fixed preparations can be stained with one dye (simple staining) or in a combined way by multiple days (multiple staining).

2.1. Differential staining.

It is the most important method for identifying bacteria, where the multi-stepped staining with different dyes allows for differentiation of different types of bacteria. The most common is Gram staining and staining acid resistant microbes.

2.1.1. Gram staining. This method was empirically established in 1884 by Danish physician and microbiologist Hans Christian Gram. Using the method developed by Gram it became possible to divide the whole bacterial world to two – gram-positive (GP) and gram-negative (GN). A multitude of modifications of this method has been later developed but they all share the same principle of operation. The method is performed in four stages:

1) a heat-fixed smear is thoroughly covered with basic dye – crystal violet (CV);
2) the smear is treated with iodine (Lugol solution), which helps to fix the stain. As a result complex of crystal-violet-iodine is formed (CV-I);
3) the smear is washed with ethanol or a mixture of ethanol and acetone;
4) additional staining with basic dye such as safranin.

While during the first and second stage the different types of microbes are not stained differently, then after third stage the GP bacteria are violet and GN colorless – because GP bacteria retain their stain after washing with ethanol. After additional staining with safranin the GN bacteria will be stained red. The key to gram-reactivity lies in the different composition of bacterial cell wall between GP and GN bacteria. Namely for GP bacteria the cell wall is composed of thick peptidoglycan (90% of cell wall) and for GN the cell wall is composed with a rather small amount of peptidoglycan (15-20%). The thick peptidoglycan will not let the CV-I dye complex be washed out from the cell, because it is larger than the CV molecule that entered the cell. Due to their thin cell wall, the CV-I complex will be washed out from inside the GN bacteria – the complex will pass through the thin cell wall and through the lipopolysaccharide outer membrane which now is made permeable due to the effects of ethanol.

Inside peptidoglycan, which is a polymer only found in bacteria, the polysaccharide chain is formed of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) connected by β-1,4-glycosidic bond.

From the latter perpendicular peptide chains connected by peptide bonds emanate. This rather rigid structure protects cell from osmotic lysis in hypotonic environment (most natural environments are hypotonic). It is still possible to crumble this peptidoglycan layer using enzyme called lysozyme which breaks the β-1,4-glycosidic bond between NAG and NAM. This crumbled peptidoglycan can no longer defend the cytoplasmic membrane inside the osmotically imbalanced environment and the cell will lyze and die. This defense mechanism that is unrelated to immune system helps humans fight bacterial infections. Lysozyme is found in most human body secretions (tears, saliva, milk etc.). Fleming discovered this cationic peptide five years before penicillin. GN bacteria have the peptidoglycan in their cell wall protected by lipopolysaccharide outer membrane. To get the effect of the lysozyme on the GN bacteria they are first treated with ethylenediaminetetraacetic acid (EDTA), which breaks the outer membrane by weakening ionic bonds.

In addition to the traditional Gram staining described earlier a company named Difco Labs has developed a 3-staged Gram staining method, which reduces the risk of removing too much dye in washing (decoloring stage) and helps to save time. Namely the decoloring and additional staining stages are moved together. Often times the so called Hucker modification is used where the first stage is sped-up by using ammonium oxalate crystal violet.

The detection of Gram-reactivity is used for example in medicinal microbiology to prescribe effective antibiotic treatment. Namely penicillin and its derivatives inhibit the peptide bond formation in murein nets meaning they inhibit the peptidoglycan synthesis inside growing cells. The latter is also inhibited by cephalosporins, vancomycin, bacitracin and cycloserine. For the reason that inside GN bacteria there is little peptidoglycan and the transport of antibiotics inside the cell is slowed or stopped by the outer membrane the GN bacteria are as a rule less sensitive to antibiotics inhibiting the synthesis of peptidoglycan.

The Gram-reactivity is determined inside young actively growing cultures, because older cultures of GP bacteria can include cells with damaged cell walls which will give false gram-reaction. There have been found generations and spores of GP bacterium *Bacillus subtilis* testing falsely as GN.

To quickly determine the Gram-reactivity one can also successfully use **KOH-lysis test** in which GN bacteria will lyze inside 3% KOH solution within 30 seconds, producing characteristic „slimy" consistency. This method has undeservedly received little attention by microbiologists, although it was first introduced already in 1938 by a Japanese called Ryu. This test certainly does not replace Gram method, because it will not give any information about cells morphological type and is easily affected by different mistakes like false lysis time or too thick bacterial suspension, which will not give viscous strains when gelling. It is also not usable for determining the Gram-reactivity of slimy bacteria.

2.1.2. Staining acid fast microbes. This combined staining method is dependent on the resistance to the decolorization by weak acids of certain types of stained cells. At first the cells are stained by heating them with carbol fuchsin, then they are decolorized with acidic alcohol solution and then additionally stained with methylene blue. Acid fast bacteria will not decolorize in the wash and keep their original stain. Acid-fastness in several *Actinomycetes* and *Mycobacterium* species which have high GC concentration correlates with the high concentration of lipids (mycolic acids) in their cell wall (up to 60% of dry weight). Mycolic acid, by bonding covalently with the peptidoglycan in cell wall, turns its surface „waxy" which will not let the bacteria be stained by Gram method. Microbiologists use this method to differentiate the pathogens from families *Mycobacterium* (can cause leprosy and tuberculosis) and *Nocardia* from other microbes.

2.2. Special staining or structural staining

This method is used to stain several different structures specific to microbial cells (such as: capsules, cell walls, endospores, flagella, nutrient storage structures)

2.2.1. Staining the capsule. Generally the term glycocalyx or capsule is used to describe polysaccharide or peptide material outside the cell proper. It is strongly bound to the cell and unlike diffuse slime it is not easily washed away. Thanks to high water content (98%) it is non-ionic and does not stain. Several pathogens and also non-pathogens can have capsules (*Klebsiella pneumoniae, Haemophilus influenzae, Bacillus anthracis, Leuconostoc mesenteroides, Streptococcus salivarius etc*.). Capsules are grouped by their size to microcapsules (not visible in light microscope, characteristic to Gram negative bacteria), macrocapsules (visible in light microscope) and to diffuse capsules which is formed by slime weakly bonded to cell. The chemical composition of the capsule varies between species. The capsule can be a polypeptide capsule (*Bacillus*), a polysaccharide capsule (*Streptococcus, Leuconostoc*) or a glycoprotein capsule. Capsule serves an important protective function as an osmotic defense barrier, defending cell from drying and unfavorable environmental conditions. Pathogens have thick capsule to protect them from phagocytosis by host. In laboratory conditions the capsule for some of those species can completely disappear. In nature the capsule helps microbes to attach to substrate. It can also be a nutrient reservoir, as it bonds different ions and molecules. The function of the capsule has also been connected to „storage room for waste products", dumping waste there can help to avoid their interference with cells metabolism.

Making a capsule smear is one of the hardest preparations. Definitely the specimen must not be fixed, to avoid the pseudo-capsule formation due to drying and on the other hand the thermal fixing can also dry the capsule itself. For staining the capsule one can use simple staining with nigrosin (which won't permeate into cell nor capsule – negative/contrast staining) or combined staining (in addition to nigrosin another (basic) dye is used which stains the cell but won't stain the capsule)

2.2.2. Staining of flagella. Flagella are characteristic to many microbes. Flagella ensure the motility of them by the way of swimming (inside liquid or semi-liquid environment) or swarming (as coordinated groups on a solid media). The latter way of motility is only applicable to those bacteria who are able to form aggregates with adjacent cells. The maximum movement speed as accounted for bacteria is 0.00015 km/h (10 cell-lengths in a second). To compare it to human these numbers would be 37.5 km/h and 5.4 body-lengths in second. The motility is determined by the size and shape, the amount of flagella and their positioning on the cell as well as the viscosity of the environment. In this way the smaller cells are usually more motile. The bacteria having helical shapes like spirochetes have an improved motility in viscous environment, based on the movement not unlike that of a corkscrew. The *bacillus* might have a single flagellum on one end of the cell and be called monotrichous *(Vibrio*) or a single flagellum on opposite ends (*Wolinella*); when two or more flagella are positioned on the same end they are called lophotrichous (*Pseudomonas*) when on opposite ends - amphitrichous (*Spirillum*) and in the case of many flagella all over the cell they are called peritrichous (*Enterobacteriaceae* and *Bacillaceae*). The flagella rotate like propellers, either clockwise or counter-clockwise. In the case of counter-clockwise rotation they push the bacterium (causing forward motility), in the case of clockwise rotation they pull the bacterium causing the cell to rotate in place. This mode of movement „on-wards intermittent with on place rotation" is for example characteristic to *E. coli*, but by the way many bacteria just move just back-and-forth. The lack of significant inertia is common to bacterial motility – in the case the flagella stop the cell stops inside 0.6 μs.

The flagella are an important taxonomic identifier, but because of their small diameter (0.02-0.04 μm) they are invisible in light microscope without special treatment. By careful

artificial thickening and staining with tannic acid and fuchsine (Leifson method) it is still possible to get a decent specimen preparation observable by light microscope. For bigger cells like these of the *Spirillum* (helical cells, with the diameter of 1.7 μm and length up to 60 μm) it is possible to differentiate flagella by the use of 1000x magnification phase-contrast microscope. Flagellin - the protein of the flagella forms cylindrical/tubular structures, whereas the growth of such structures is performed by adding new flagellin sub-units which move from inside the tube to the end of it. Lately the method suggested to visualize flagella is based on fluorescent protein-dye NanoOrange which binds to flagellin (Grossart method), which allows to examine the flagella by fluorescent microscope

2.2.3. Staining endospores. Commonly only one endospore forms inside a bacterial cell and this process of **sporulation or sporogenesis** is initiated by unfavorable environmental conditions. Species that can form endospores are found inside seven bacterial genera whereas mainly these are comprised of rod-shaped gram-positive bacteria such as from genera *Bacillus, Clostridium, Desulfotomaculum* etc. These are phylogenically close species with small GC content. Endospores can be considered as „dormant bacterial cells". Their thick walls protect them from unfavorable environmental conditions (extreme temperatures, lack of water, toxic components, radiation etc.) and allow them to survive for very long time (millions of years). It is not quite clear what kind of biochemical reactions trigger this process, but in its first step a plasma-membrane forms around replicated DNA and a small amount of cytoplasm. Following that a pre-endospore with two-layered membrane forms. Between these layers a peptidoglycan layer is synthesized. Outside the membrane a thin peptide layer „exosporium" which is responsible for the spores' resilience towards toxic compounds. The thermoresistance of endospores is proportional to the dipicolinic acid content inside them. The low water content (10-30% of vegetative cell) ensures a metabolically inactive state in spite of the existence of metabolites and enzymes inside endospore. In addition there is DNA in endospores, a small amount of RNA, some ribosomes and some low-molecular weight compounds, especially dipicolinic acid (15% of dry weight), which is lacking in vegetative cells. All these are important to re-start metabolism in the case of spore germination. For the reason that the endosopores are resistant to temperature, chemicals etc. they do dictate the conditions of sterilization. For example to destroy them special thermal treatment is needed (15-20 min, 121 °C) called autoclaving. Hereby it must be mentioned that endospores can be used as a biological indicator of the efficiency of the sterilization process. For that endospores capable of germination are determined in sample before and after the treatment of the sample under some conditions. After plating the colonies one can perform direct counting of the endospores by phase contrast microscopy. Incubation is rather time consuming and for that reason L-alanine, L-asparagine or glucose is used to speed up the germination. To determine the viability of endospores (EVA – *endospore viability assay*) the amount of dipicolinic acid released while autoclaving is also successfully used.

The diameter of the endospore can be the same as its parent cell, but can also be a little bit smaller or larger. Depending on the species the spore can be situated terminally, sub-terminally or centrally. The most common type of spore is centrally positioned, ovally shaped and with the width of parent cell – type common to bacilli. After the spore inside the cell is mature the cell walls lyze and the cell is destroyed – the spore is released. To identify spores it is important to know conditions that trigger sporulation. According to literature Mn-ions increase the number of sporulating cells. According to our data the ideal medium to observe spore formation is R2A agar, which is used to determine the amount of heterotrophic organisms as a generic growth medium. Apparently the Mg-ions which are contained in R2A medium are too a sporulation inducing factor. In this way it is possible to quickly determine the microbe's capability to produce endospores, as already after the 20 hours of growth on the R2A medium it is possible to see cells containing endospores (**sporangia**) or already released

endospores. Inside LB medium (a culture medium) one would usually not see a single sporulating cell in such time window.
The thick spore coat that causes many of the spore's resistances is also a reason why spores will not ordinarily stain. For example spores will not be stained by Gram and we can then only detect them if they are still connected to the cell. To stain endospore a weak cationic dye is used - malachite green, which is induced to endospore by thermal treatment and is later easily removed from cytoplasm by washing with water. After that an additional staining with safranin is performed, where after we see malachite green endospore on the red background of the sporangium's cytoplasm (Schaeffer-Fulton method). A very quick and convenient way to detect endospores is to examine cells by phase contrast microscope.

2.2.4. Staining of nutrient storage structures. It is characteristic to many microbes especially those from the genera *Bacillus, Clostridium, Pseudomonas*, to form nutrient storage structure in response to certain environmental conditions - granules of lipids, polyhydroxybutyrate (PHB), polyphosphates, polysaccharides and other such materials. These materials are stored in cell mainly when the microbe's growth is limited or inhibited by some growth substance. When conditions suitable for growth are restored they can quickly be plugged back into cell metabolism as the source of carbon or energy. Some storage materials are common to most bacteria and some are only found in some species – in this way the storage materials are a taxonomic identifier. The capability to store these materials gives a microbial cell an advantage in surviving unfavorable environmental conditions

Lipid resembling substances (PHB). In yeasts and micro-fungi the lipid storage substances are held in vacuoles as neutral fats, which can be observed by phase contrast microscopy due to different refraction of light. In prokaryotes different sized polyhydroxyalkanoate (PHA) polymer granules (with a monomer structure of -CHR-CH_2-COO-) exist as an equivalent to storage fats. The most common form of this storage material is poly-β-hydroxybutyrate (PHB), which has a methyl group in parallel chain. The capability to accumulate PHB is an important taxonomic criteria. First bacteria in which PHB granules were isolated and identified was *Bacillus megaterium*. The fact that they are stained by lipophilic dyes has caused them to be confused with drops of fat. Purified PHB itself does not stain with lipophilic days so it is speculated that granules of PHB are connected to other lipids. PHB functions as carbon and energy store inside the cells and is produced by bacteria when inside an environment rich in carbon and poor in nitrogen. By this imbalanced growth where the biosynthesis of nucleic acids and proteins is inhibited the PHB content can reach up to 90% of the cell's dry weight. Species from genera *Mycobacterium, Bacillus, Azotobacter, Beggiatoa etc.* belong to the group of bacteria synthesizing this storage substance. During last years works have been published which show that the PHB is involved with endospores and sporulation. Currently the PHA polymers are used as the feed stock to generate biodegradable plastics

Meta-chromatin/volutin/polyphosphate granules. Plenty of microbes are capable of storing polymetaphosphate storage substance called volutin in their cells as a response to different environmental conditions. The process is specifically pronounced in the case of sulfate deficiency inside the environment rich in phosphates. When the sulfate stores are restored the polyphosphate granules disappear from the cell quickly as the phosphate is incorporated into nucleic acids. First these granules were discovered inside the cells of *Spirillum volutans* and therefore they are called volutin granules. Staining by Loeffler method, the methylene blue binds strongly with phosphate crystals and in few minutes they are stained dark blue on the background of light blue cytoplasm. Staining volutin with basic blue dye a metachromatic effect is achieved – the prior blue color turns to red. The methods of staining volutin are based on its bad solubility in acids.

Polysaccharide granules. From polysaccharides starch, glycogen and granulose (which is by structure similar to starch) are collected inside microbial cells as storage materials. The last one is specifically stored by clostrides. Glycogen as a carbon and energy storage is among microbes more common than starch. It is collected by bacilli, enterobacteria and *Micrococcus luteus* but also by yeasts and other micro-fungi. Detecting polysaccharides from bacteria is impossible by light microscopy as they are quite uniformly spread around cytoplasm. Polysaccharides from yeast cells can be determined by using Lugol solution which the starch and granulose turn blue and glycogen brown. The blue color characteristic to starch is then created thanks to iodine reacting with the coil structure of the amylose which is composed of glucose monomers bound by α-1,4-glycosidic bonds. Glycogen is similar in its composition to amylopectin (in addition to α-1,4-glycosidic bonds there are many α-1,6-glycosidic bonds which causes the polymer chain to branch). Glycogen stains well in acidic environment.

QUESTIONS

1. Why are bacterial cells badly visible in light microscope and how can one make them more visible?
2. What kind of dyes are used when staining microbes?
3. How will microbes stain with cationic dyes when in acidic environment and why?
4. In which conditions do acidic dyes stain better and why?
5. How do the methods of staining with acidic or basic dyes differ?
6. The principle behind negative staining.
7. What inhibits the staining of live cells?
8. What kind of function does peptidoglycan have in a bacterial cell?
9. What is lysozyme and what does it affect?
10. Does lysozyme affect archaea and mycoplasmas?
11. Where in nature is lysozyme found and why there?
12. What kind of effects has lysozyme on Gram-negative and on Gram-positive bacteria?
13. What importance plays the fixing of cells on the microscope slide?
14. What is the advantage of using chemical fixing?
15. What is peptidoglycan?
16. What is Gram reactivity based on?
17. What function does the ethanol solution serve in the Gram staining?
18. What are the main steps in the Gram staining and how are GN and GP bacteria colored during each of these steps?
19. What step from the original Gram method can one skip?
20. Why is it important to use Lugol solution when doing staining by Gram?
21. Why can one not use staining by Gram to stain acid resistant microbes?
22. Can differences in cell wall structure cause different Gram reactivity?
23. How do the yeasts stain by Gram?
24. Do the acid resistant microbes fall into the category of Gram-negative or Gram-positive microbes due to the virtue of their cell wall composition?
25. What is the most common morphological type for bacteria?
26. What function has the capsule to the cell and how does one stain it?
27. Why is a smear of capsule not fixed?
28. What is the function of copper(II) sulfate in the staining of the capsule?
29. Why does the capsule not stain?
30. What is the function of endospores in bacteria?
31. How does one identify endospores in cells?
32. How does the preparation of endospore look after staining by Gram and why?
33. Why is it easy to wash the malachite green out from the cell?
34. Why can one not observe the flagella of bacteria by light microscopy and how can one still observe them?
35. Compare the flagellal diameter to the diameter of a bacterial cell and to the resolution of the light microscope**.**
36. What causes the accumulation of nutrient storage substances inside cell and why is such accumulation an important matter of research?
37. What is the function of PHB granules?
38. What is volutin?
39. What is the main polysaccharide nutrient storage substance in microbes?

PRACTICAL WORK AND RESULTS

A. STUDYING LIVE CELLS

Work no. 1. PREPARING A SIMPLE WET MOUNT AND LIVE STAINING BY FICKER

Material to be studied: cell suspension of ***Saccharomyces cerevisiae*** (for two first smears) and ***Micrococcus luteus*** culture from solid media (for third smear).

1) Prepare 3 clean grease-free microscope slides;
2) with a pipette or a plastic loop place two drops of microbial suspension on the slide or homogenize some culture taken from a solid media inside a distilled water drop on the slide by using a plastic loop. We make two smears per one slide – one with non-treated microbial suspension and then the same with treatment;
3) the drops of microbial suspension should then be covered with cover slips in a following way – first one edge of the cover slip must be made to contact the drop, then it must be slowly felled upon the drop;
4) after covering the smears with cover slips, we do the following for both slips. We place a small drop of neutral dye next to the edge of the cover slip (neutral red or methylene blue, 1% solutions in water) and make it move under the slip by placing a piece of filter paper next to the opposite edge of the slip. After a few minutes of staining the wet mount smears will be examined by light microscopy in a dry system and then be described and analyzed.

The treatments are following:

1) treat the **yeast** suspension before placing it on the slide for 15 min at 100 °C;
2) add a drop of 0.1% cetyltrimethylammonium bromide (CTAB) to **yeast** suspension and let it work for a few minutes;
3) add a drop of lysozyme to **bacterial** suspension (50 mg/ml) and let it work for a few minutes.

Work no. 2. "HANGING DROP" MOUNT TO STUDY BACTERIAL MOTILITY

Material to be studied: ***Erwinia carotovora*** (***Pectobacterium carotovorum***) – non-aggregated peritrichous bacteria, ***Bacillus coagulans*** – aggregated peritrichous bacteria, ***Pseudomonas mendocina*** – monotrichous, ***Janthinobacterium lividum*** – 1 polar and 2-3 lateral flagella, cell suspensions in logarithmic growth phase or an enrichment culture from pond water

1) Prepare a larger cover slip and a special microscope well depression slide. The edges of the depression will be smeared with a small amount of vaseline to avoid drying up the drop;
2) transfer a small amount of microbial suspension in the **center of the cover slip** by using micro-pipette;
3) place the well depression slide onto the cover slip so that the depression covers the drop, press upon it gently to form a small hermetic chamber;
4) turn the whole slide over quickly and gently. A hanging drop mount is now prepared inside which a small drop hangs over a depression by the surface tension. Be careful to make sure that the drop would not touch the edges of the depression!
5) Examine the mount inside a dry system by first finding the edge of the drop by using small magnification, then refocusing when using larger magnification. **Turn down the light!**
6) Compare the modes of movement depending on the placement of the flagella.

B. STUDYING A FIXED MOUNT

Work no. 3. STAINING BY GRAM

Material to be studied: a choice to take one bacterial culture per two bacterial sets which differ from each other by the effect of lysozyme:

I set – lysozyme-negative: ***Escherichia coli, Enterobacter aerogenes, Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas mendocina;***

II set – lysozyme-positive: ***Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Rhodococcus pyridinovorans, Deinococcus radiodurans.***

1) Make a suspension from both cultures upon one microscope slide. Let it air-dry and fix them thermally: drag the slide held by tweezers few times through the flame so that by touching it to the back of the hand it feels hot;
2) stain the fixed smear for **1 min** with Hucker crystal violet and wash the smear with distilled water for few seconds;
3) cover the smear with fresh Lugol solution for **1 min.**;
4) wash again with distilled water;
5) decolorize the smear up to **30 s** with 96% ethanol. **Do not exceed the time limit!**
6) Wash the smear 5 s with distilled water and dry the excess water carefully with strips of filter paper;
7) stain the smear 20-30 s with 0.25 % safranin;
8) wash, dry and microscope the smear in oil immersion system (place a drop of oil on the smear and use oil immersion objective);
9) analyze the smear and explain why did they stain differently.

Work no. 4. QUICK METHOD FOR DETERMINING GRAM-REACTIVITY
Material to be studied: the same bacterial cultures you used in the last work.

1) Place 1-2 drops of freshly made 3% KOH solution on the microscope slide;
2) suspend 0.5-1 min the cell cultures studied in previous work inside the drop;
3) use the tip of inoculation needle to rise suspension 1-2 cm from the drop – observe if viscous threads form (GN-bacteria) or do not (GP-bacteria).

Work no. 5. STAINING ENDOSPORES
Material to be studied: sporulating (induced) bacterial cultures of ***Bacillus sphaericus***, ***Bacillus subtilis*** or ***Bacillus circulans***.

- **Schaeffer-Fultoni method**

1) Cover air dry thermally fixed smear with 5% aqueous solution of malachite green. Warm **carefully** over flame for **10 min**. Keep an eye on that the smear would **not dry (add malachite green to cooled down slide as needed) or boil**!
2) Wash the cooled smear thoroughly with distilled water and stain additionally for 20 s using 0.25% safranin;
3) wash the smear with distilled water until colorless. Dry and microscope using oil-immersion system;
4) evaluate the results of the staining and explain.

- **Staining by Gram** (as per work no. 3)

Work no 6. STAINING FLAGELLA BY HEIMBROOK
Material to be studied: cell cultures in logarithmic growth phase or an enrichment culture from pond water

1) Pipette a drop of (50-100 µl) distilled water onto the plate containing bacteria and incubate it on the table for few minutes so that the bacteria can swim into the drop. Use the **inoculation loop** to transfer the material onto the drop of water (10 µl) on the (really clean) slide.

   OR

   In the case of enrichment culture from pond water just pipette 10 µl on the slide.
2) Cover the smear with cover slip and add a drop of flagellum dye (**APPENDIX I)** under one edge and absorb it under the slip by placing a strip of filter paper next to opposite edge. Remove the excess dye with filter paper;
3) let the dye work for **5-15 min**;
4) place a drop of immersion oil on the cover slip and examine the smear with 100x objective. **Be very careful when focusing as the cover slip is really brittle! Avoid the contact of the object and the dye!**

Work no. 7. STAINING VOLUTIN
Material to be studied: cell suspension from yeasts ***Saccharomyces cerevisiae*** or ***Schizosaccharomyces pombe***.

1) Make and air-dry smear from the cell suspension and fix it in flame;
2) stain the fixed smear for 10 min with 1% solution of toluidine blue where a drop of 1% sulfuric acid is also added;
3) wash, dry and microscope in oil-immersion system;
4) observe dark blue granules on the background of light blue cytoplasm, observe the metachromatic effect of the granules.

Instead of toluidine blue one can also use an older Loeffler methylene blue solution.

Work no. 8. STAINING GLYCOGEN
Material to be studied: cell suspension of the yeast ***Saccharomyces cerevisiae.***

1) Make a suspension of yeast cells on the slide and acidify it with a drop of 1% sulfuric acid;
2) add a drop of Lugol solution on the slide and cover it with the cover slip (in case of excess suspension remove some of it with the use of a strip of filter paper);
3) microscope using high magnification dry system;
4) observe the existence of reddish-brown granules inside the cells.

Work no. 9. STAINING POLYHYDROXYBUTYRATE GRANULES
Material to be studied: bacterial cultures of ***Bacillus subtilis*** *and* ***Bacillus mycoides***.

1) On one slide prepare a thermally fixed smear from two different bacteria;
2) stain for 30 min with Sudan black and wash with 96% ethanol. **Do not wash with water!**
3) Perform additional staining with safranin (30 s) and wash with distilled water;
4) microscope in oil-immersion system and observe the existence or lack thereof of black granules in the cells.

Work no. 10. DIFFERENTLY SHAPED BACTERIA

Material to be studied: A sample taken from the filter used to purify drinking water – a suitable environment for iron oxidizing bacteria. These bacteria oxidize Fe^{2+} found in the water to Fe^{3+}, after which the precipitated $Fe(OH)_3$ is easily observed in microscopy by the virtue of its strong reddish-brown color and therefore there is no need to stain these preparations.

1) Make an **air-dry smear** and microscope using oil-immersion system to find ***Gallionella ferruginea*** cells and their inorganic stems composed of uniquely twisted filaments that can be up to 400 µm long;
2) find from the same smear a different iron oxidizing bacteria - filamentous ***Leptothrix ochracea***, the cells of which reside inside strong filamentous sheath.

Work no. 11. PLEOMORPHISM

Material to be studied: differently aged cells of ***Rhodococcus pyridinovorans***.

1) Prepare a thermally fixed smear from the differently aged cells of the culture on the both sides of the slide
2) perform simple staining with a basic dye such as crystal violet, wash, dry and study in immersion system;
3) find the differences between differently aged cells from the same bacterial culture.

Work no. 12. STRESSED BACTERIA

Material to be studied: cells of ***Janthinobacterium lividum*** grown at different temperatures.

1) Prepare a thermally fixed smear from one side of the slide out of the bacterial culture grown at 22 °C and to the other side out of the bacterial culture grown at 30 °C;
2) perform simple staining with a basic dye such as crystal violet, wash, dry and study in immersion system;
3) find the differences between cells from the same bacterial culture grown at different temperatures.

Work no. 13. MICROORGANISMS INVOLVED IN THE FERMENTATION OF FOODSTUFF

Material to be studied: different yogurts, fermentation liquid of sauerkraut and naturally fermented pickles (cucumbers).

1) Make a heat fixed smear from chosen foodstuff (in case of yogurt make a suspension inside a drop of distilled water)
2) perform simple staining with a basic dye such as crystal violet, wash, dry and study in immersion system;
3) analyze what different shapes and sizes of microorganisms exist inside studied foodstuff.

C. NEGATIVE STAINING

Work no. 14. A PREPARATION FROM DENTAL PLAQUE OR POND WATER
Material to be studied: oral microflora gathered with sterile swab from dental pocket and from between the teeth or enrichment culture of pond water.

1) Make a suspension inside the drop of a distilled water out from the dental plaque or set a drop of pond water on the end of the slide;
2) add a drop of 1% nigrosin to the suspension and mix;
3) smear the stained suspension evenly around the slide using another slide (a greyish layer stays on the slide) and dry;
4) microscope using immersion system;
5) examine and analyze the preparation.

Work no. 15. SPECIMEN PREPARATION OF CAPSULE
Material to be studied: ***Pseudomonas fluorescens*** or ***Bacillus licheniformis*** bacterial cultures with slimy consistency that have been grown on solid media.

It is possible to stain a capsule smear by using simple or combined staining.

Capsule smear will not be fixed!

- **Simple staining** is to be performed with nigrosin by making a cell smear on one side of a grease free microscope slide. Out of which a smear is prepared according to the procedure outlined in work 14. Study in immersion system and analyze.

- **Combined staining** of the capsule using modified **Anthony method**:

1) on a grease free microscope slide prepare a microbial smear out of the cells with slimy consistency;
2) add a drop of crystal violet (1% **aqueous solution**);
3) carefully smear the preparation around using another slide;
4) stain (dry) for 5-7 min;
5) wash the preparation with **some** drops of 20% $CuSO_4x5H_2O$ solution, by letting the solution flow down on a tilted preparation slide;
6) dry the smear, and examine the area that is decolorized by copper sulfate by using an immersion system. Analyze!

II MICROBIOLOGICAL MEDIA, PREPARATION AND STERILIZATION

A. MEDIA

Microbes like all other living organisms need diverse selection of inorganic and organic compounds for their growth. One of the most important substances is water (comprising 70-80% of the cell), which due to its polar structure is an ideal biological solvent. For a microbe to grow either in nature or in a laboratory, both chemical and physical conditions necessary for growth need to be provided. The prior describe the chemical composition of growth medium, existence of suitable C- and energy source and necessary growth factors. Microbial cell needs energy for the biosynthesis of cellular substances. While **chemoorganotrophs** (metabolic type of most bacteria) obtain energy for degradation of organic molecules through fermentation or respiration processes, **chemolithotrophs** get energy from oxidation of inorganic ions.

Microbial growth medium must be a complete medium, meaning it must contain all necessary **macro-** (C, O, N, H, P, S, K, Mg, Ca, Fe) and **micronutrients** (Mn, Zn, Mo, Cu, Ni, V, Na, Se, Si, W etc.) These elements appear in water as inorganic ions, monomers or polymers. Each element has a certain physiological role to play in a cell. **Carbon** as the most important element is necessary in a living cell for building organic compounds and it constitutes nearly half of the dry weight of a bacterial cell. When **heterotrophs** obtain C from organic compounds, like proteins, carbohydrates and lipids, **autotrophs** use CO_2 as a C-source. **Nitrogen** makes up 14% of the dry weight of the cell, being a component of amino acids, nucleic acids, nucleotides, coenzymes and vitamins. Many bacteria degrade proteins using the obtained amino acids for *de novo* synthesis of proteins, and most bacteria can use nitrogen from ammonium ions or some also from nitrate ions. Nitrogen fixing bacteria (e.g. *Rhizobium* and *Azotobacter*) who can use air nitrogen for the synthesis of cellular substances are also known. **Phosphorus** is included in nucleic acids, nucleotides, phospholipids and lipopolysaccharides, making up 3% of the dry weight of the cell. Other macronutrients constitute significantly smaller part of the dry weight of the cell (1% or below). Microbes need **micronutrients** in very small amounts, therefore they are added to the media as concentrated solutions and immediately before pouring out the media. Most micronutrients are cofactors of enzymes.

Growth factors are natural organic substances that organism can not synthesize itself, thus these need to be added to the medium. For example, purines, pyrimidines, amino acids and vitamins are growth factors. The nutritional requirements of different microbes are so different that there are no universal media where all microbes could grow. Some bacteria don't need addition of growth factors to the medium (e.g. *E. coli*), as for others they are obligatory (e.g. *Lactobacillus*). Mutant bacterial strains requiring certain growth factors are called **auxotrophs**, while wild type strains with no such requirements are called **prototrophs**.

When preparing media, the accordance of **pH** to the recipe must checked. Often indicator dyes are added to the media (Table 1), allowing to assess microbial growth by the color change as a result of the pH change. Indicator must not be toxic to the microbes. Usually, the concentration of indicator does not exceed 0.01%. Excessive pH changes during the growth are prevented by adding buffering components to the media (phosphates, carbonates, proteins). If pH is significantly differing from the initial pH, microbial cells will lyze.

Depending on the composition, media are classified into 3 groups:

1. **Natural media** are with undefined composition, like milk, meat broth, berry and fruit juices etc. These are used to maintain cultures or to produce biomass.
2. **Synthetic media** are with chemically defined composition and are prepared using distilled water and pure chemicals (C, N, P, S and growth factors which the cell is not capable of synthesizing). Synthetic or defined medium is a **minimal medium** if it contains a certain C- and energy source for microbial growth. Using this type of medium presumes knowledge of nutritional requirements of the microbe. These media are appropriate for elucidating nutritional requirements of microbes and for several physiological researches.
3. **Complex media** have undefined chemical composition, meaning in addition to minerals they contain undefined additives: yeast, meat or plant extracts. Only few microbes can use these proteinous substances without prior degradation, but degradation products resulting from partial acidic or enzymatic hydrolysis of the protein, like peptone or tryptone, can be utilized by many microbes. **Peptone** is a hygroscopic water soluble powder produced by an enzymatic hydrolysis of animal proteins, consisting of shorter and longer peptides, amino acids, nitrogen bases and mineral salts. It is added to the medium 1-20 g/l and it can also be a C-source for the microbe. Also, an enzymatic hydrolysate of milk protein casein called **tryptone,** which is especially rich in tryptophan can be used as an undefined additive. Product resulting from acidic hydrolysis of casein (**casamino acids**) is also successfully applicable as a source of amino acids (except tryptophan, content <0.01%), N, S and P. **Yeast extract**, a water soluble extract of lyzed yeast cells, contains group B vitamins, organic N- and C-compounds and it is usually added to the media 0.5-5 g/l. Complex media are used to culture unknown or fastidious microbes.

Table 1. Indicator dyes

Indicator dye	pH transition range	Color in acidic environment	Color in basic environment
Brilliant green	0.0-2.6	yellow	green
Bromocresol green	3.8-5.4	yellow	blue-green
Bromocresol purple	5.2-6.8	yellow	purple
Bromophenol blue	3.0-4.6	yellow	blue
Bromothymol blue	6.0-7.6	yellow	blue
Methyl red	4.4-6.4	red	yellow
Neutral red	6.8-8.0	red	yellow
Phenol red	6.8-8.4	yellow	red

Media are also classified according to **functional use** or **application**.

1. Miscellaneous physiological, genetic and biochemical test use **minimal medium** containing minimal amount of nutrients and a carbon source essential for microbial growth.
2. In microbiological research, often specific microbial groups need to be differentiated. For this purpose, favorable conditions for a specific microbial culture to be isolated are generated (appropriate C- or N-source, pH, oxygen regimen etc.) and substances to inhibit the growth of other microbes are added (dyes, antibiotics, acids, etc.). This type of media are called **selective media**.
3. **Differential media** allow fast distinction of one microbial strain or group from others by clear differentiating characteristics. This type of media are most often used in medical and sanitary microbiology. For example, Endo medium is both selective and differential. Basic fuchsine, decolorized due to the presence of sodium sulfite, acts as a pH indicator in the medium. Complex of sodium sulfite and basic fuchsine inhibits the growth of gram-positive microbes. The medium is also differential due to lactose. Lactose fermenting bacteria generate a metabolic intermediate acetaldehyde which binds sodium sulfite, thus released

fuchsine turns the bacterial colonies red. Colonies of microbes fermenting lactose most effectively are dark red and have characteristic metallic luster, whereas bacteria not fermenting lactose form colorless and pale pink colonies on Endo medium.
4. Similarly to selective media **enrichment media** generate conditions for faster growth of a specific group of microbes (e.g. isolation of air nitrogen-fixing microbes on N-free medium). By repetitive inoculation from one enrichment medium to another, it is possible to rapidly increase the number of the fastest growing bacteria in that medium. Enrichment media do not contain agents suppressing the growth of other microbes, thus the growth of unwanted groups of microbes is not inhibited. The first enrichment media were introduced by M. Beijerinck and S. Vinogradski.

Based on **consistency**, media are classified as liquid, semisolid and solid media. The easiest way to prepare a **liquid medium** is to use dehydrated nutrient broth containing meat extract and peptone. Meat extract contains water soluble nutrients, vitamins and minerals cooked out of the muscle tissue. An example of a complex medium supporting the growth of many microbes is a tryptic soy broth, which contains tryptone and plant peptone as the main ingredients. The former is a powder produced by enzymatic hydrolysis of casein, the latter is obtained by enzymatic hydrolysis of soybean meal. **Solid media** are prepared by adding gelling agents to the liquid media. Agar (1.5-2%) and gelatin (10-20%) are used as gelling agents. For certain tests (e.g. bacterial motility tests) **semisolid media** need to be used, where the concentration of a gelling agent is low (0.2-0.3%). Silica gel plates immersed with nutrient solution could also be used as a solid medium. This method is adopted if the presence of organic substances inhibits the growth of microbes (chemolithotrophic bacteria).

Agar is an excellent gelling agent as it is microbiologically inert, hydrolyzed only by few microbes isolated from the seawater (e.g. *Cytophaga fermentans*, *Flavobacterium flevense*). Agar melts at water boiling temperature and solidifies at 42 °C. Agar is prepared from the red algae and is marketed both as sheets and a powder form. Agar media are never poured out while very hot to prevent development of unwanted water condensation. When agar media are autoclaved, it needs to be taken into account that under pH 5.5 agar hydrolyzes and will not set after sterilization. If a medium with extreme pH value is prepared, water agar (agar + distilled water) is prepared separately and the medium is mixed after sterilization. Chemically, agar is a galactan, a polymer composed of galactose residues.

Gelatin is a gelling agent derived from animal bones, cartilage, sinews and other collagen-rich tissues. Gelatin liquefies at 25 °C, thus can't be used at higher temperatures as a gelling agent. Another disadvantage of gelatin is that it is degraded by microbes producing proteolytic enzymes. Nowadays, gelatin-containing media are mostly used to detect proteolytic properties of microbes (gelatin liquefaction is an important diagnostic characteristic).

B. STERILIZATION

Sterilization refers to **complete** elimination of all forms of microbial life from instruments, media and other materials. Almost sterile conditions are not appropriate for microbiological work. Manipulations to reduce microbial number are carried out on both inanimate objects (**disinfection –** fire, water, phenols) and living tissues (**antiseptics** – iodine, alcohol) and these techniques are applied in microbiology to sterilize surfaces and instruments or for safety precautions.

The **effect** of antimicrobial agent on exponentially growing cell culture can be the following:
1. **Bacteriostatic** (also **fungistatic**), which refers to conditions where the growth of bacteria (fungi) is inhibited. For example, freezing and action of several chemicals, especially dyes.

2. **Bactericidal** (also **fungicidal**), which refers to the lethal action of various chemical and physical factors to microbial cell. These effects on cell structures are irreversible. All sterilization techniques are bactericidal in principle, except filtration.

Antimicrobial action can be related to:

- disruption of cell wall or inhibition of its synthesis (chemical);
- modification of permeability of cytoplasmic membrane (chemical);
- damaging proteins and nucleic acids (chemical, thermal, irradiation);
- inhibition of enzymatic apparatus (thermal, chemical);
- inhibition of synthesis of proteins and nucleic acids (chemical).

Efficacy of sterilization can depend on:

1) physical and chemical properties of the environment to be sterilized (pH – thermal treatment is more effective in acidic environment, high carbohydrate concentration significantly increases thermal resistance);
2) microbial life form (vegetative cells are more sensitive compared to spores);
3) physiological state of the cell (young actively metabolizing cells are more easily affected than growth arrested cells);
4) microbial species (pseudomonads grow well even in the presence of some compounds used for chemical sterilization, e.g. phenol).

Advantage of using one or the other sterilization technique is decided based on the physicochemical properties of the material to be sterilized and also research tasks.

Sterilization can be divided into thermal and cold sterilization.

Thermal sterilization comprises: heating in a flame or flame sterilization, sterilization with dry hot air, fractional sterilization i.e. tyndallization with hot flowing steam and sterilization with steam under pressure. Thermal sterilization is relatively low-cost and easily controlled. **Pasteurization and boiling in water do not completely sterilize a material. Boiling in water** destroys vegetative cells in 15-30 minutes, but not heat resistant endospores. Boiling is used to clean different instruments from microbes.

In **pasteurization**, the liquid is mostly heated to a temperature under 100 °C, also having a denaturizing effect. The method does not ensure sterility, but is widely applicable for elimination of pathogenic microbes form foodstuff in conditions where higher temperature could spoil the taste. Mostly short-term (71 °C 15 s) or longer (63 °C 30 minutes) thermal treatment is used. Nowadays, food processing industry (e.g. dairy industry) also uses ultra-high pasteurization, where the temperature of treated material is raised very high (135-140 °C) for a short period of time (1-2 s).

Cold sterilization comprises treatment with certain chemicals, filtration and irradiation with x-, γ- or UV-radiation.

1. THERMAL STERILIZATION

Thermal sterilization kills microbes by irreversible denaturation of microbial enzymatic proteins. Different groups of microbes have different thermal sensitivity, characterized by a **thermal death point** (**TDP**), which is the lowest temperature at which all microbes are killed in a liquid medium in 10 minutes The second factor characterizing the time needed for sterilization is a **thermal death time** (**TDT**) – a minimal time needed to kill all bacteria in a liquid medium at a given temperature.

In practice, thermal sterilization is divided into moist and dry heat sterilization:

1.1. Moist heat sterilization, which combines high temperature and moisture destroys microbes by coagulating their proteins due to the disruption of tertiary structure H-bonds. Moist heat is faster and more effective compared to dry heat.

1.1.1. Fractional sterilization i.e. tyndallization is a stepwise sterilization in flowing steam for 20 minutes at 100 °C during three consecutive days. At intermissions of heating the material to be sterilized is kept in an incubator at 37 °C to germinate the spores. This time-consuming and nowadays not very important method is used to sterilize such materials that do not stand heating in overpressure.

1.1.2. Autoclaving is a sterilization with hot steam under pressure and is the most commonly practiced sterilization method in a laboratory. Thermal treatment of the material takes place in a saturated water vapor at a pressure above atmospheric pressure (overpressure) in special hermetically sealed autoclaves. This enables to raise the water boiling temperature above 100 °C. The temperature in an autoclave is proportional to the overpressure (atm):

For example, at 0 atm 100 °C
0.5 atm 112 °C
1.0 atm 121 °C
1.5 atm 128 °C

Bactericidal effect of autoclaving is caused by the high temperature of the water vapor. Next, simplified principle of autoclaving is presented.

Autoclave consists of two connected chambers: sterilization and steam chamber. Steam chamber is supplied with a tap through which it is filled with distilled water. Water level in an autoclave can be controlled by water level indicator on steam chamber. The water is brought to boil in a steam chamber and if the valve between two chambers is closed, the pressure starts to increase to the necessary level, which is regulated by the contact manometer connected to the steam chamber. As the valve is opened, steam – ejecting the air - is led to the sterilization chamber and through air outlet to a vessel filled with water. When the sterilization chamber is filled with steam, the air outlet is closed and the generated overpressure in the chamber is registered by the connected manometer. Autoclaving regimen is fixed on contact manometer and after reaching certain predetermined pressure the autoclaving time is being registered. After proper sterilization time, autoclave is switched off from the power circuit and let to cool. When overpressure is nearly zero, first air outlet and then autoclave door are carefully opened, letting the material to further cool and dry.

The efficacy of autoclaving depends on the bulkiness of the material to be sterilized, sterilization time and autoclave load. For example, if sterilization of 10 l of medium autoclaved in one container takes 1 hour, sterilization of 1000 times 10 ml of medium takes 10-15 minutes. Autoclave should not be filled too tightly – the steam needs to reach all objects. Most certainly it is necessary to avoid air pockets in sterilization chamber caused by insufficient deaeration.

1.2. Dry heat sterilization, which destroys microbes due to the oxidation of their components at high temperature, has lower penetrating power than moist heat sterilization.

1.2.1. Flame sterilization – inoculation needles, spatulas, forceps, microscope slides and cover slips, flask openings etc. The objects are heated in the upper third part of the flame where the temperature is the highest.

1.2.2. Dry heat air sterilization. Special dry hot air sterilizers are used. Usually, the materials are heated at 170 °C for 2 hours, as the protein structures are more resilient to dry thermal sterilization. This technique is used to sterilize powders, viscous solutions and glassware.

2. COLD STERILIZATION

2.1. Chemical sterilization can be applied if thermal sterilization is unsuitable due to thermolability of the materials. For example, it is used to sterilize devices, some plastics etc. Different chemical agents have different mechanisms of action. For example, phenol affects cell wall and cytoplasmic membrane causing denaturation of proteins; alcohols affect

cytoplasmic membrane and cause denaturation of proteins, but do not affect endospores. To determine the sterilizing effect of various chemicals, **phenol coefficient** (PC) is used which compares antimicrobial effect of a chemical to phenol under standard conditions. PC is calculated by dividing the highest dilution of a compound which is still lethal to the highest lethal dilution of phenol in 10 minutes treatment. To evaluate cell culture growth, the cells are incubated for 48 hours at 37 ºC. If the treatment with the chemical in question has no effect on growth at a dilution 1:450 and with phenol – 1:90, the PC of the tested compound is 450/90=5. In this case, the tested compound is 5 times more effective than phenol.

Chemical sterilization with gases are carried out in special hermetic gas autoclaves. For example, sterilization under pressure with ethylene oxide vapors is becoming an important cold sterilization method. Ethylene oxide is very toxic to virus particles, bacterial and fungal cells and also to thermoresistant bacterial endospores as it covalently binds to cell proteins. The ability to easily penetrate packing materials makes it especially effective sterilant. Ethylene oxide is relatively low-cost, non-corrosive as opposed to other toxic compounds and the residues are easily removable. In practice, a non-flammable and incombustible mixture of ethylene oxide and carbon dioxide (1:9) is used.

2.2. Mechanical sterilization is a removal of microbes from solutions or gases by filtration through biologically inert fine porous nitrocellulose filters. Those membrane filters are manufactured by companies like Millipore, Sartorius, Advantec, Nuclepore etc. and the diameter of the pores is always marked. This sterilization technique is used for such solutions and media that contain thermolabile compounds. To sterilize small volumes of thermolabile compounds Swinney filters or syringe filters with different pore sizes are used.

2.3. Sterilization by irradiation. Sterilizing effect of irradiation depends on the radiation wavelength, intensity and duration. Electromagnetic radiation with wavelength below 300 nm is lethal to the cells. This includes UV, γ and x-ray radiation. Two types of radiation with lethal effect on microbes are distinguished – non-ionizing and ionizing.

2.3.1. Non-ionizing radiation with less energy is UV-radiation having lethal effect on cells at wavelengths between 210-300 nm. Due to low penetration power it is suitable for sterilization of air and surfaces. The most effective bactericidal region in UV spectrum is 250-260 nm, related to the direct influence on the DNA of exposed cells. Irradiation with that wavelength causes covalent bonding of adjacent pyrimidines in one DNA strand and the formed thymidine dimers disturb correct DNA replication in cell division.

2.3.2. Ionizing radiation (x-rays and γ-radiation) is a radiation with shorter wavelength (below 100 nm) and thus higher energy, and better penetration power. The effect on DNA is indirect, meaning that the radiation ionizes water and forms highly reactive hydroxyl radicals which react with cellular organic compounds, including DNA. The resistance of different bacterial strains to ionizing radiation is different. For example, GN bacterial genus *Pseudomonas* is rather sensitive, whereas GP bacterial genus *Micrococcus* is more resistant. This sensitivity depends greatly on the ability of the microbial cell to metabolically neutralize free radicals. It needs to be taken into account that spores are highly resistant to ionizing radiation as they have low water content. For that reason, destroying spores with irradiation takes longer time. This method is widely used in pharmaceutical industry and production of sterile laboratory equipment.

QUESTIONS

1. Which elements are necessary for the cell growth?
2. Why is it necessary to add growth factors to the microbial growth medium?
3. How is it possible to evaluate the growth of microbes in certain medium?
4. How are media classified based on their composition?
5. How is synthetic medium different from complex medium?
6. How are media classified based on their functionality?
7. Which media group (both composition and functionality) does minimal medium with phenol belong to?
8. Is it possible for a microbial cell to grow only on peptone?
9. What is yeast extract and could it be a source of vitamin C?
10. What is differential medium? Give some examples.
11. How is selective medium different from enrichment medium?
12. Which substances are responsible for the selectivity of a microbial growth medium?
13. How are media classified based on to their consistency?
14. What physical and chemical properties of agar enable its use as a gelling agent in solid media?
15. Why is gelatin not appropriate as a gelling agent in media?
16. Why shouldn't agar media be poured out while too hot?
17. Why are agar plates kept up side down after they are solidified?
18. Why is agar autoclaved separately for media with extreme pH?
19. Which factors causing cell death affect cell wall, plasma membrane, proteins and nucleic acids?
20. Which thermal sterilization technique is the most effective? Why?
21. Does pasteurization of foodstuff ensure sterility and why are foodstuff pasteurized?
22. What affects the value of thermal death point?
23. What is the bactericidal factor of autoclaving?
24. What does the efficacy of autoclaving depend on?
25. What are the cold sterilization techniques?
26. How are the mechanisms of action of ionizing and non-ionizing radiation different?
27. Why is it necessary to open the Petri dish when exposing it to UV-radiation?
28. How are thermolabile solutions sterilized?
29. Are GP or GN bacteria more sensitive to chemical sterilization?
30. Calculate the PC of lysol to *S. aureus* cells if the dilution of phenol 1:20 and dilution of lysol 1:300 kill all cells in 10 minutes?
31. How can the resistance of *Pseudomonas*, *Bacillus* and *Mycobacterium* genera to several sterilization techniques be explained?

PRACTICAL WORK AND RESULTS

1) Prepare a medium for the microbiology experiments, optionally according to guidelines in APPENDIX II and supervisors instructions;make preparations for sterilization of various microbiological materials using dry and moist sterilization techniques;
3) get acquainted with the construction and guidelines of the autoclave and autoclave both prepared materials and media;
4) after autoclaving, prepare agar deeps and slants in test tubes and sterile plates in Petri dishes. If needed, carry out cold sterilization of single components of the medium.

When preparing a medium, please note:
1) use the chemicals and components indicated by the recipe; if there are differences in the quantity of some substances, recalculations are needed;
2) weighing of dehydrated media (highly hygroscopic) must be done quickly and carefully to avoid dispersal and inhalation of volatile powder. Protective mask needs to be worn when weighing powder concentrates containing toxic indicator dyes;
3) use distilled water free of heavy metals to prepare media;
4) use flasks with appropriate size to prepare media, so the flask is never filled to the brim;
5) the medium is brought to the boiling temperature to dissolve agar;
6) if the pH of the medium is 5 or below it is never autoclaved together with agar;
7) the pH of the medium must correspond to the recipe. The pH is checked with pH-meter before addition of agar or indicator dyes and autoclaving;
8) only **clean** spatulas, scoops and weighing paper can be used for weighing reagents;
9) the balance and its surroundings need to be **clean** after weighing;
10) all flasks and test tubes containing solution or medium need to be marked.

Many media exist as commercial dehydrated powders, manufactured mostly by Difco Laboratories, Baltimore Biological Laboratory (BBL) and Becton Dickinson. These media are supplied with ingredients list and instructions for preparation. Recipes for both commercial and self-mixed media used in microbiology practicum are listed in APPENDIX II.

III ASEPTIC TECHNIQUES FOR INOCULATION

A. INOCULATION METHODS

Microbes are transferred by inoculation from one medium to another with properly sterilized instruments (inoculation loops, needles, sticks, spatulas, automatic pipette tips). The work of a microbiologist is facilitated by using sterile single-use plastic inoculation loops and needles. To avoid contaminating medium with airborne foreign microbes, the inoculations are usually carried out in inoculation chambers pre-sterilized with UV-radiation. Nowadays, mostly laminar flow hoods are used for this purpose, where laminar air flow (air carried through filter systems makes up a sterile air curtain between the front panel of the hood and the experimenter) protects the inoculation from contamination with the external air and the experimenter from microbial infection. If there is no inoculation chamber, some inoculations can be done on a laboratory benchtop but then always with the aid of a Bunsen burner or an spirit lamp. Inoculation is carried out in the proximity of the flame and in that part of the laboratory where air movement is minimal, whereas benchtop surface is first disinfected with 70-80% ethanol or lysol (cresols-K-soap aqueous solution).

Inoculation instruments and methods are chosen according to the aim of the work and the consistency of the inoculum. Thus, inoculating loop is used in case of making a **streak-plate** to transfer microbes from one solid medium to another, inoculation needle is used for making a stab culture by thrusting it deep into the medium. Inoculation loop is also good for transferring the inoculum from liquid culture to solid or liquid medium. A streak-plate technique is often used to isolate pure cultures, to inoculate cultures in separate sectors of the plate, to cultivate a small quantity of microbes etc.

For quantitative analyses, automatic pipettes with removable sterile tips are used to inoculate solid or liquid medium with a liquid culture. To inoculate a plate with a liquid microbial suspension (usually 0.1-0.2 ml) the plate is thoroughly smeared with the inoculum using a sterile spatula – this is called a **spread-plate** method. The spatula is dipped into ethanol and drawn through the flame for sterilization (**NB! The spatula should not be held in the flame to avoid breaking it!**). When ethanol has burned off the spatula, the lid of the Petri dish is partially opened and the spatula is cooled against the cover of the Petri dish or on the microbe free part of the medium. Cooled spatula is used to spread the inoculum over the whole surface of the agar plate, until the surface becomes dry. If a microvolume (10 µl) is used, it may be left unspread – this is called a **drop-plate** method. This technique allows to save plates as one plate can be inoculated with up to 12 drops. It is important to avoid moving the plate after using this technique until all the liquid has absorbed into the medium (approximately 30 minutes). This method is certainly unsuitable for counting colonies of swarming bacteria. Optimal number of colonies in one drop is 3-30.

Pour-plate method can also be used to inoculate a solid medium with liquid inoculum. In this case, 15-20 ml of agar medium at a temperature of about 50 °C is added to a small amount of microbial culture in a Petri dish, the dish is closed with a lid and the contents are immediately mixed by moving the plate on the benchtop with circular motions. If the spread-plate method allows us to distribute the inoculum evenly on the surface of the plate, the pour-plate method allows us to distribute the inoculum evenly throughout the whole volume of the medium and colonies (visible mass of microbial cells) grow both inside the medium (lense-shaped) and on the surface. Spread-plate method favors microbes tolerant to oxygen, while pour-plate method helps to monitor the growth of microbes more sensitive to oxygen but less sensitive to temperature. In the latter case, the inoculum size could be increased (usually 0.5-1 ml). All above-mentioned plating techniques are widely used for counting the microbes.

In addition to these methods, **replica-plate** method is carried out using different sterilized imprints.

Inoculation techniques:

1. Mark a sterile test tube, flask or plate (the lid) containing medium with your initials and the name of the inoculating culture;
2. sterilize inoculation loop in the upper third of the flame of a Bunsen burner (~1500 °C) holding it tilted relative to the flame. For sterilization the whole metal part of the loop needs to be heated until glowing hot.

A. Inoculation of a medium in a test tube:

3. take the test tube with inoculum into the free hand (stir to homogenize in case of liquid culture);
4. remove the cap from the tube with inoculum, so that the cap will be placed between little finger and palm of the same hand as inoculating loop and heat the mouth of the tube in the flame;
5. cool the loop against the inner wall of the test tube and pick the inoculum with it;
6. remove the inoculation loop from the test tube and heat the mouth of the test tube in the flame before recapping it;
7. open the test tube with the sterile medium (the cap will be taken between the little finger and the palm) and heat the mouth of the test tube in the flame before inserting the loop. If inoculating solid medium, slide the loop over the agar surface, if inoculating liquid medium, shake the loop in the medium;
8. sterilize the mouth of the test tube before recapping and the loop before putting it away to completely incinerate the microbial material on it.

B. Inoculation of a medium in a plate:

3. cool the sterile inoculation loop at the edge of the sterile plate;
4. open the test tube with inoculum (heat the mouth of the test tube before inserting the loop) or plate and take some microbial cells on the loop;
5. remove the inoculation loop from the test tube and before recapping heat the mouth of the test tube in the flame or replace the plate back to the lid;
6. if inoculating a sterile medium in a Petri dish, place the dish up side down on a benchtop with the free hand, then lift the bottom to the vicinity of the flame and carry out streaking;
7. close the plate and sterilize the inoculation needle before putting it away;
8. incubate the inoculated plate bottom up to prevent water condensation.

B. PREPARING DILUTIONS

The number of bacteria in a sample may reach several millions, so generally it needs to be diluted before quantitative analysis. Mostly sterile normal saline (0.9% NaCl) or distilled water is used for making dilutions. Depending on the task and available sterile instruments, different dilution schemes can be used. Sequential dilutions of the original sample result in dilution series, most commonly decimal or tenfold dilutions are prepared. If the inoculum size is low, 1.5 ml eppendorf tubes are most convenient to use. Having the total volume of a sample and distilled water 1.0 ml, meaning 0.1 ml of the first and 0.9 ml of the second, enables adequate mixing in 1.5 ml eppendorf. **The sample must be vortexed and the pipette tip replaced with a new sterile one before making the next dilution.**

The reciprocal of the dilution is called dilution factor and is mathematically expressed as:

$$\boldsymbol{Dilution\ factor} = \frac{\boldsymbol{sample\ volume\ (ml)\ or\ weight\ (g) + diluent\ volume\ (ml)}}{\boldsymbol{sample\ volume\ (ml)\ or\ weight\ (g)}}.$$

Counting the number of microbial colonies grown on the plate enables to calculate the number of cells in the initial sample, taking into account **dilution factor** and **inoculum size**. It is expressed as colony-forming units (CFU) per 1 ml initial sample:

$$\boldsymbol{Microbial\ number}\left(\frac{\boldsymbol{CFU}}{\boldsymbol{ml}}\right) = \frac{\boldsymbol{number\ of\ colonies\ (CFU) \times dilution\ factor}}{\boldsymbol{inoculum\ size\ (ml)}}.$$

Optimal number of colonies per one plate should be in the range 30-300 because less than 30 colonies don't give statistically reliable information about the microbial number. More than 300 colonies on a plate could give a smaller number due to overgrowth and also the counting is more complicated.

QUESTIONS

1. What is the difference between spread-plate and pour-plate methods?
2. Is it possible that a bacterium grows in a streak-plate but not in pour-plate?
3. When is it practical to use a stab culture?
4. When is streak-plate method used on an agar slant or a plate?
5. Why is agar cooled down to about 50 °C before it is poured into a Petri dish with pour-plate method?
6. Why are plates incubated in a thermostat inverted (bottom up)?
7. In which cases is it better to use an inoculation needle for streaking?
8. Why are test tube and flask mouths heated before and after inoculation?
9. Which inoculation technique and medium would you use for cultivating a large microbial mass?
10. Why is it necessary to make dilutions in order to determine the bacterial number?
11. What is a dilution factor?
12. What does a decimal dilution mean?
13. How should a dilution series be prepared in order to obtain a final dilution of 10^{-10}?
14. Why is it necessary to use a new pipette tip for making each following dilution?
15. What is the optimal number of colonies to be counted on a plate?
16. What does the term CFU mean?

PRACTICAL WORK AND RESULTS

Before inoculation mark the test tubes and Petri dishes containing sterile media with your initials, group number, name of the microbe used for inoculation and if needed, incubation temperature. Use the bottom of the plate for marking, but write the text on the edges of the bottom. Incubate the plates **bottom up**!

A. STREAKING AN AGAR SLANT WITH AN INOCULATION LOOP (from agar slant)

Work no. 1. EVALUATING THE USE OF CITRATE BY THE BACTERIA

On **Simmons agar slant** inoculate one of the following bacterial species following the recommendations of the supervisor: ***Escherichia coli, Enterobacter aerogenes, Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas mendocina, Bacillus subtilis, Bacillus licheniformis, Bacillus circulans, Bacillus coagulans, Bacillus sphaericus, Micrococcus luteus, Proteus vulgaris, Deinococcus radiodurans, Acinetobacter baylyi, Rhodococcus pyridinovorans, Erwinia carotovora, Ralstonia eutropha, Serratia marcescens, Janthinobacterium lividum*.** Incubate at a temperature of 30 °C, except ***Bacillus coagulans*** – 55 °C, ***Janthinobacterium lividum* – RT** (room temperature).

Work no. 2. EVALUATING THE USE OF UREA BY THE BACTERIA

On **Christensen agar slant** inoculate one of the previously mentioned bacterial species following the recommendations of the supervisor. Incubate at a temperature of 30 °C, except ***Bacillus coagulans*** – 55 °C, ***Janthinobacterium lividum* –** RT.

B. MAKING A STAB CULTURE WITH INOCULATION NEEDLE TO A AGAR DEEP (from agar slant)

Work no. 3. DETERMINING THE METABOLIC TYPE OF THE BACTERIA

On **LB agar deep** inoculate one of the previously mentioned bacterial species following the recommendations of the supervisor. Incubate at a temperature of 30 °C, except ***Bacillus coagulans*** – 55 °C ***Janthinobacterium lividum* –** RT.

C. STREAKING AN AGAR PLATE WITH INOCULATION LOOP (from liquid culture)

Work no. 4. THE INFLUENCE OF pH OF THE MEDIUM TO MICROBIAL GROWTH

Streak a plate of **nutrient agar with glucose** with one of the following pHs: 3, 5, 7, 9, 11. Divide the bottom of the plate into 5 sectors and mark with the names of inoculating strains according to supervisor's suggestions (4 bacterial strains and 1 yeast). Each group should make inoculations from the two following selections:

I selection: ***Hansenula polymorpha, Escherichia coli, Bacillus circulans, Pseudomonas fluorescens*** and ***Rhodococcus pyridinovorans;***

II selection: ***Saccharomyces cerevisiae, Bacillus licheniformis, Acinetobacter baylyi, Enterobacter aerogenes, Proteus vulgaris*.**

Incubate at 30 °C.

D. INOCULATING A BROTH WITH A PLASTIC INOCULATION LOOP (from liquid culture)

Work no. 5. THE EFFECT OF TEMPERATURE ON THE GROWTH OF MICROBES
Inoculate one of the following suspensions of microbes to LB broth according to the suggestions of the supervisor and incubate at appropriate temperatures:
E. coli at temperatures: 4, 20, 30, 37, 42, 50 °C;
B. coagulans: 4, 20, 30, 37, 42, 50, 55, 60 °C;
J. lividum: 4, 20, 30, 37 °C;
P.fluorescens: 4, 20, 30, 37, 42 °C.

E. MAKING A SPREAD-PLATE USING DRIGALSKI SPATULA

Work no. 6. THE EFFECT OF UV-RADIATION ON THE CELL VIABILITY
Study material: overnight bacterial culture (10 h) of ***E. coli*** and ***Deinococcus radiodurans.***
Each student makes one inoculation of a dilution suggested by the supervisor.

1) The dilutions suitable for inoculation and later UV-radiation exposure times (marked with an asterisk) are presented in the methodical table below;
2) into sterile eppendorfs, make decimal serial dilution of a microbial suspension suggested by the supervisor. The suspensions need to be mixed properly when making the dilutions;
3) make one **nutrient agar spread plate** using inoculum size of 100 µl;
4) after inoculation place the marked plate (strain name, dilution, UV exposure time) on a respective stand;
5) the open dishes are irradiated with UV-lamp at 20 cm distance, covered with the lid and incubated overnight at 30/37 °C (this will be done by the supervisor).

Lethal action of UV-radiation on different bacterial cultures

Bacterial strain	**Time (s)**	**Dilution**							
		10^0	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**
E. coli	0						*	*	*
	5		*	*					
	30	*							
Deinococcus radiodurans	0					*	*	*	
	30					*	*	*	
	120			*	*				

F. POUR-PLATE METHOD

Work no. 7. DETERMINING THERMAL DECIMAL REDUCTION TIME
Study material: unpasteurized milk. **Each student makes one inoculation according to the supervisor's suggestions.**

1) Eppendorfs with milk are incubated in a thermomixer at certain temperature, **63 °C or 72 °C for a predetermined period**. Place **all eppendorfs at once** into the thermomixer for a thermal treatment. Before starting the stopper, check if the set temperature has been reached in a control eppendorf filled with water;
2) when treatment time ends, place the samples back onto the ice box.
3) **there is 1 ml eppendorf on the ice box for each student**, marked with temperature, treatment time and dilution;
4) to determine the number of bacteria, make a dilution series for obtaining a dilution marked on the Eppendorf;
5) **0.5 ml** of a milk sample or an appropriate dilution of it is transferred to a sterile Petri dish, then un-gelled **MPCA** medium at 45 °C is added, mixed with circular movements to obtain homogenous inoculum, and let to set and incubated at 37 °C for 24 h.

Survival of microbial population at different temperatures in unpasterurized milk

Temperature	Time (min)	Dilution						
		10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	0					*	*	*
63 °C	10				*	*		
	20		*	*				
	30		*	*				
72 °C	5			*	*			
	10		*	*				
	15	*	*					

IV EFFECT OF ENVIRONMENTAL CONDITIONS ON MICROBIAL GROWTH

Microbial growth depends on both physical (temperature, radiation, pH, gaseous environment, osmotic pressure) and chemical factors (nutritional conditions, chemicals, co-culture effects). Optimal growth conditions are not the same for different microbes. A microbiologist is interested in those conditions that would result in the largest biomass. Medics, on the other hand, are interested in suppressing microbial growth to prevent and cure microbial diseases. This control over the growth is complicated by the great adaptability of microbes. Below, some environmental conditions affecting microbial growth are explored.

A. TEMPERATURE

Microbes can grow in a wide temperature range (below 0 °C and above 100 °C). The physiological effect of temperature on a microbial growth is closely related to the direct influence of temperature on the activity of microbial enzymatic apparatus. At low temperatures the enzymatic activity is reduced and thus growth rate is also reduced. Microbes that can grow at higher temperatures have thermoresistant enzymatic apparatus due to the stabilizing effect of several metal ions and higher content of hydrophobic amino acids in the proteins. Also, thermophilic microbes have more saturated fatty acids in their membranes. On the other hand, at low temperatures membranes with that kind of structure become waxy and thus non-functioning. To avoid this, bacteria that grow in cold conditions have more unsaturated fatty acids in their membranes. Due to differences in the cell membranes prokaryotes are able to grow at much higher temperatures than eukaryotes. For example, archaeal membranes consist of stable ether lipids that allows them to live in extreme conditions (very high temperature, very high or low pH).

Microbial growth rate is highest at **optimum temperature**. Microbial growth can occur between a **maximum temperature** (close to optimum) and a **minimum temperature** (much lower than optimum). An example is given to illustrate how much minimum-optimum-maximum temperatures (°C) of some species can differ, although these temperatures may vary depending on other environmental conditions (pH, nutrients):

Bacterium	Minimum t°	Optimum t°	Maximum t°
Pseudomonas fluorescens	4	25-30	40
Staphylococcus aureus	6	30-37	46
Thermus aquaticus	40	70-72	79

Microbes are classified into 5 groups depending on optimum growth temperature: psychrophilic and phsychrotrophic (thrives at low temperatures), mesophilic (thrives at moderate temperatures), thermophilic (thrives at high temperatures) and hyperthermophilic microorganisms. Minimum-optimum-maximum temperatures of these microbial groups are depicted in Table 2.

Table 2. Classification of microbes by growth temperatures

Group	Minimum t°	Optimum t°	Maximum t°
psychrophiles	<0	5-15	20
psychrotrophs	0	20-30	35
mesophiles	15-20	30-40	45
thermophiles	40	55-65	72
hyperthermophiles	67	90-100	113

It is important to note that the lowest possible temperature even for psychrophilic microbes to grow at is the freezing point of water, meaning 0 °C for pure water and -2.5 °C for seawater. Although low temperature inhibits microbial growth, it may not be lethal. Substances, like glycerol etc., that lower water freezing point and prevent formation of ice crystals in the cell, are added to the growth media (final concentration not below 10%) to preserve microbes at very low temperatures (-70 °C or below) and ensure long-term viability.

As described in chapter II, boiling is the easiest way to reduce the number of microbes in a liquid culture. It must be taken into account that not all microbes have the same temperature tolerance. Some microbes can withstand even 100 °C for some time. These microbes are called **thermoresistant** and as a rule can form endospores. When a material containing microbes is treated with higher temperature, the number of microbes is reduced logarithmically, whereas thermal death time (TDT) of the culture depends both on the size of the initial population and treatment temperature. **Decimal reduction time** (DRT or D with a temperature marked as a lower index), is the **time required at a certain constant temperature to reduce the number of viable cells by a factor of ten** (an order of magnitude) in the sample. DRT is used for quantitative evaluation of thermoresistance of the microbial population. For example, if initial population of 10^6 microbes is heated for one minute at certain temperature and $9x10^5$ cells are destroyed and $1x10^5$ cells survive, and after treatment for 2 minutes $9.9x10^5$ cells are destroyed and $1x10^4$ cells survive, then D value is 1 minute. Mathematically, D is determined from the equation:

$$\boldsymbol{t = D \times (logN_0 - logN_t)},$$

where D is decimal reduction time (min) at certain temperature, N_0 – initial population size; N_t – population size at time t; t – exposure time (min).

To find D and TDT values experimentally at certain temperature, the culture is incubated at that temperature and samples are periodically drawn to determine the number of viable cells. The data collected are used to construct a plot, where log-values of the viable cell numbers are plotted on y-axis and the time points on x-axis. A straight line will be fitted through these plotted points. For the determination of D value two other types of lines will be drawn. First, two lines parallel to x-axis that start from y-axis logarithm markings (denoting cycles in which the number of living cells changed 10 times) and end on the fitted line, and second two lines parallel to y-axis that start from endpoints of previously drawn lines and end on the time-axis (x-axis). Obtained time interval is the value of D. TDT will be determined by extension of the fitted line to cross the x-axis, the point where the number of living cells is 0. TDT can be also found by multiplying the starting population's cell number with obtained D value:

$$\boldsymbol{TDT = logN_0 \times D}.$$

D value of the population of sample depends on physiological state of the cells and environmental conditions, like pH (acidic environment promotes cell death). These described

parameters are most important in food industry, as minimal thermal treatment time is aimed to preserve taste properties.

In addition to affecting microbial growth rate, temperature has a direct effect on the ability of microbes to produce pigments. Most of sea psychrotrophs are pigmented, whereas none of the known thermophiles can produce pigments. For example, the production of pigment prodigiosin in bacterium *Serratia marcescens* is directly dependent on the temperature. At higher temperatures, the enzyme catalyzing the synthesis of this pigment is repressed. The function of this pigment is unknown, although the chemical structure is known and it contains pyrrolic structure similar to chlorophyll.

B. OXYGEN CONSUMPTION

One environmental factor greatly affecting microbial growth is oxygen. Molecular oxygen, vital for the existence of some microbes, is a poisonous gas to the others. The toxicity of oxygen is related to the generation of harmful byproducts, peroxides and superoxide, which by reacting vital structures cause cell death. Several enzymes in microbes function to neutralize reactive oxygen species.

1. **Superoxide dismutase** – degrades toxic superoxide radicals generated in aerobic respiration to molecular oxygen and hydrogen peroxide:

$$\mathbf{O_2^{-}. + O_2^{-}. + 2H^{+} \rightarrow H_2O_2 + O_2\uparrow}$$

2. **Catalase –** degrades toxic hydrogen peroxide generated in aerobic respiration to water and molecular oxygen:

$$\mathbf{2H_2O_2 \rightarrow 2H_2O + O_2\uparrow}$$

3. **Peroxidase –** degrades hydrogen peroxide to water without generating oxygen:

$$\mathbf{H_2O_2 + 2H^{+} \rightarrow 2H_2O}$$

Microbes are classified into following groups according to how they react to oxygen:

a) **obligate aerobes** require O_2 to grow and oxygen serves as a terminal electron acceptor in respiration. They have both catalase and superoxide dismutase. Growth can occur either on the surface of the agar deep or on the upper part of it where sufficient amount of oxygen can diffuse. Microbes undergoing aerobic respiration belong into this group (e.g. *Micrococcus, Pseudomonas* etc).

b) **facultative anaerobes (aerobes)** can tolerate oxygen deficiency by switching to fermentation or anaerobic respiration. The best growth still occurs in aerobic conditions, thus they gather mostly on the upper part of the agar deep. The bacteria in this group have both catalase and superoxide dismutase (e.g. *Escherichia, Staphylococcus* etc.).

c) **obligate anaerobes** can grow only in oxygen-free environment as they lack enzymes detoxifying reactive oxygen species. The growth can occur only on the bottom of the agar deep. They metabolize either by fermentation or anaerobic respiration (e.g. *Clostridium, Bacteroides*).

d) **aerotolerant anaerobes** preferentially grow in anaerobic conditions, but oxygen does not inhibit them. They have superoxide dismutase, which partially neutralizes oxygen toxicity, but lack catalase. They grow uniformly throughout the agar deep. They metabolize by fermentation (e.g. *Lactobacillus, Streptococcus* etc.).

e) **microaerophiles** are aerobes that require lower oxygen concentration (2-10%) compared to oxygen levels in air (20%). This is due to the lack or low activity of catalase and superoxide dismutase. Growth occurs in the upper part of the agar deep, but not on the surface. These microbes mostly undergo aerobic respiration, less often anaerobic respiration (e.g. *Spirillum*, *Helicobacter* etc.).

C. pH

Microbial growth is greatly affected by the environmental pH - the concentration of H^+ ions (-log[H^+]) - which affects proteins and other charged molecules in the cell. Most microbes grow well at pH 5.5-8.0, but some can also grow in extreme conditions, like strongly acidic (pH 1.5-5.5) and strongly alkaline (pH 8.5-11.5) conditions. These microbes are called **neutrophiles, acidophiles** and **alkaliphiles**, accordingly. Most bacteria are neutrophiles, whereas microfungi prefer acidic environment (pH 4-6).

Optimal pH for growth is reflected in extracellular pH. Although microbes can generally grow in rather wide pH range, their tolerance is limited. Some microbes (e.g. bacterial blood parasites) can have quite narrow optimal pH range, some (e.g. enterobacteria) have it quite wide. Drastic pH changes are lethal to microbes, as it disrupts the plasma membranes and inhibit enzymatic reactions. Environmental pH also affects solubility of growth substrates in water, which in turn determines their availability to microbes. Although the environmental pH can vary a lot, intracellular pH is rather stable and **close to neutral**. Bacterium will die if intracellular pH drops below 5. This is due to cytoplasmic membrane which is relatively impermeable to protons and the proton excess is pumped out.

Microbes often need to adapt with environmental changes. Microbes themselves can alter the environmental pH with their metabolic products and this has to be taken into account for laboratory cultivation. Fermenters produce organic acids from carbohydrates, chemolithotrophs like *Thiobacillus* oxidize reduced sulfur compounds to sulfuric acid. Some microbes turn the environment alkaline as they degrade amino acids and release ammonia.

For example, citrate utilizing bacteria alkalify **Simmons** medium and this is easy to observe with indicator bromothymol blue color change in the medium. Citrate as the sole C- and energy source is utilized, one of the end products CO_2 is released and combines with sodium ions and water, resulting in alkaline product – sodium carbonate. As it accumulates, pH increases. Complex medium (**Christensen's agar**) is used to identify urea degrading microbes, as ammonia released from urea increases the pH:

$$\mathbf{CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2}$$

For example, *Proteus vulgaris*, *Bacillus pasteurii* and *Sporosarcina ureae* constitutively, independent from the environment, produce enzyme urease, the synthesis of which does not require induction with carbamide. Synthesis of urease is neither repressed by the end-product ammonia. The aforementioned bacteria degrade all urea present in the environment to ammonia and they can tolerate resulting increase in pH rather well. This simple test helps to identify and differentiate bacteria with similar biochemical properties.

Buffers are added to the media to reduce the pH changes caused by metabolic activity of growing bacteria. Buffer most often used is phosphate buffer composed of weak acid ($H_2PO_4^-$) and it conjugated base (HPO_4^{2-}). As the environment is acidified, protons combine with conjugated base,

$$\mathbf{H^+ + HPO_4^{2-} \leftrightarrow H_2PO_4^-}\,.$$

As alkalinity increases, OH^- ions are neutralized with protons donated by weak acid

$$\mathbf{OH^- + H_2PO_4^- \leftrightarrow HPO_4^{2-} + H_2O}.$$

Most media are composed of amino acids, peptones and proteins, which can act as natural buffers due to amphoteric properties.

D. UV-radiation

The maximal bactericidal effect of UV-radiation is at 265 nm. As described in chapter II, UV-irradiation directly affects DNA. Immediate exposure with visible light after UV treatment can reduce the bactericidal effect of UV, because bacteria and eukaryotes have flavin-dependent enzyme – photolyase, activated by light and degrading C-C bonds between pyrimidine dimers. This reaction opposite to the lethal action of UV is called **photoreactivation**. Lethal action to the cell depends on irradiation time, power of the UV-lamp, physiological state of the cell and the presence of radiation blocking covers.

QUESTIONS

1. What is the physiological effect of temperature directly related to and what shows it?
2. How are microorganisms classified according to temperature sensitivity?
3. Does temperature classification also reflect microbe viability?
4. Why are optimal and maximal growth temperature distinguished?
5. What is characterizing the possible minimum growth temperature?
6. Why is *Bacillus megaterium* not classified as thermophilic, although its thermal death time is rather long?
7. What group are most microbes responsible for spoiling food classified to according to growth temperature?
8. What is thermoresistance and what makes it undesirable?
9. What is decimal reduction time (DRT)?
10. How is DRT different from thermal death time?
11. If DRT of the culture during autoclaving is 1.5 min, how much time does it take to kill 10^6 cells? What happens to the cells if sterilizing is discontinued after 9 min?
12. Find DRT of a microbe if initial inoculum is $5x10^6$ spores and treatment for 10 min at 121 ^oC results in 30 viable cells?
13. Why is oxygen toxic to some microbes?
14. Which enzymes are responsible for degrading toxic oxygen species?
15. How are microbes classified according to oxygen requirements?
16. How would you prepare agar deeps to study microbial oxygen consumption?
17. Characterize the growth of aerotolerant anaerobes in agar deep.
18. Which type of metabolism is characteristic to facultative anaerobes?
19. What is the difference between obligate anaerobes and microaerophiles?
20. Which group is anaerobic respiration characteristic to and how is it different from aerobic respiration?
21. How can aerobe *Pseudomonas aeruginosa* grow in anaerobic conditions?
22. What is the critical point in environmental pH changes?
23. How are microbes classified according to the pH suitable for growth?
24. What is the optimal pH for the growth of bacteria and what is for microfungi?
25. Explain how can intracellular pH remain constant when extracellular pH changes?
26. What inhibits cell growth at non-optimal pH?
27. How do microbes change the pH of their growth environment?
28. Why is it necessary to add buffers to the growth media of microbes?
29. How can *Helicobacter pylori* survive in human stomach?
30. How does UV-radiation kill microbial cells?
31. Why does UV-radiation kill vegetative microbial cells, but not endospores?
32. Which UV region is most lethal to microbes?

PRACTICAL WORKS AND RESULTS

Fill the tables below based on the cultivations done during **III topic**.

A. EVALUATION OF UTILIZATION OF DIFFERENT C-SOURCES AND OXYGEN CONSUMPTION

Based on the cultivations done during III topic works no. 1 and 2, evaluate the utilization of citrate and urea (+) or lack thereof (-) based on indicator color change (see Table 1 chapter II).

Based on the cultivation done during III topic work no. 3, observe microbial growth at different parts of the medium column and determine the metabolic type of the microbe: obligate aerobe, facultative or aerotolerant anaerobe.

Microbe	**Citrate (Simmons slant agar)**	**Urea (Christensen slant agar)**	**Growth in deep agar (metabolic type of the microbe)**
Acinetobacter baylyi			
Arthrobacter sp.			
Bacillus circulans			
Bacillus coagulans			
Bacillus licheniformis			
Bacillus sphaericus			
Bacillus subtilis			
Deinococcus radiodurans			
Enterobacter aerogenes			
Erwinia carotovora			
Escherichia coli			
Janthinobacterium lividum			
Micrococcus luteus			
Proteus vulgaris			
Pseudomonas fluorescens			
Pseudomonas mendocina			
Pseudomonas putida			
Ralstonia eutropha			
Rhodococcus pyridinovorans			
Serratia marcescens			

B. EFFECT OF MEDIUM pH ON MICROBIAL GROWTH

Based on the cultivations done during III topic work no. 4, evaluate the microbial growth visually by the strength of the streak line. Use evaluation scale, where (-) marks no growth, (+) marks weak, (++) strong and (+++) very strong growth. Analyze the growth of one strain simultaneously at all pH-s!
Classify the analyzed microbes according to the optimum growth pH:
acidophile (pH 1.0-5.5)
neutrophile (pH 5.5-8.0)
alkaliphile (pH 8.5-11.5)

Microbe	pH					Classification
	3	5	7	9	11	
Hansenula polymorpha						
Escherichia coli						
Bacillus circulans						
Rhodococcus pyridinovorans						
Pseudomonas fluorescens						
Saccharomyces cerevisiae						
Bacillus licheniformis						
Proteus vulgaris						
Acinetobacter baylyi						
Enterobacter aerogenes						

C. EFFECT OF TEMPERATURE ON MICROBIAL GROWTH

Based on the cultivation done during III topic work no. 5, evaluate bacterial growth visually according to the opalescence of the suspension. Use evaluation scale, where (-) marks no growth, (+) marks weak, (++) strong and (+++) very strong growth. Analyze simultaneously the cultivations of one strain!
Classify the bacteria according to the growth temperatures as shown in Table 2.

Microbe	Growth temperature (ºC)								Classification
	4	20	30	37	42	50	55	60	
E. coli									
B. coagulans									
J. lividum									
P. fluorescens									

D. EFFECT OF UV-RADIATION ON CELL VIABILITY

Count the colonies grown from the cultivation done during III topic work no. 6, calculate cell number in the initial sample (CFU/ml) and transfer the data to an *Excel* table. From the data of the whole group, calculate and evaluate the percentage of UV-radiation lethality (according

to the equation below) for different aged *Bacillus subtilis* cells at the same irradiation times, and for *E. coli* and *D. radiodurans* cells at different irradiation times.

$$\boldsymbol{lethality}\ \% = \mathbf{100} - (\frac{\boldsymbol{N_t}}{\boldsymbol{N_0}}) \times \mathbf{100}\ ,$$

where N_t marks the number of cells in suspension after UV-treatment and N_0 marks the number of cells in the initial suspension before UV-treatment.

Evaluation:

E. DETERMINING THERMAL DECIMAL REDUCTION TIME

1) Count the colonies grown from the cultivation done during III topic work no. 7 and calculate the bacterial number (CFU/ml) in the initial sample;
2) fill out an *Excel* table with the data of the whole group and draw a plot describing thermal resistance of milk microflora on both temperatures using semi-logarithmic scale. Cell numbers go on the logarithmic y-axis and incubation times go on the x-axis;
3) from the plot, find thermal reduction times (D) and death times (TDT) corresponding to both treatment temperatures.

V DETERMINING THE CELL NUMBERS AND MASSES OF MICROBES

Doing microbiological work, it is often necessary to quantify cell numbers or biomass in a microbial population, -consortium or community. Microbial number refers to the number of microbial cells and biomass – amount of microbial cell material per unit of volume or mass, usually per one milliliter or gram of sample. It is possible to determine the biomass in case of all types of microbes, including long filamentous microorganisms (e.g. microfungi) whose precise number is difficult to determine. Number is often used to characterize bacterial and yeast populations and communities.

Four different microbial life forms are distinguished: live – active and culturable, dormant – inactive and culturable, live – active and unculturable, dead – inactive and unculturable. Methods used for determining both number and biomass are based on either culturability or metabolic activity of the microbes or their general number (total number/biomass).

Prior determining microbial number or biomass comes sampling, for example from microbial population grown in a laboratory or different environments (soil, natural water, foodstuff, drinking water etc.). The sample must be analyzed instantly, if possible, or fixed with suitable methods. The fixing agent preserves the cell number unchanged and prevents cell lysis and deformation. Most commonly used fixatives of bacterial samples are highly reactive formaldehyde (final concentration 2%) and glutaraldehyde (final concentration 1%), which combine with proteins and inactivate them. Glutaraldehyde is used to differentiate between phototrophic and heterotrophic microorganisms, as formaldehyde may obscure chlorophyll autofluorescence.

Thus, process of determining cell number can be divided into three stages: (1) sampling and sample treatment (fixing and diluting depending on the need), (2) cultivation of the sample or sample dilution or making a preparation (3) and counting cells or colonies.

A. DETERMINING CELL NUMBER

1. INDIRECT METHODS FOR DETERMINING LIVE CELL NUMBER

1.1. Plating samples on agar medium or plate count method

Conventional plate count method is the most commonly used method for indirectly determining live cell number. The method presumes that one colony grows from each live cell spread on a plate and the inoculum is homogenous and doesn't contain microbial aggregates. These presumptions are not always met, as cells close to each other or cell aggregates could form one colony. This is the reason for expressing such numbers as colony-forming units (CFU-s) per volume or mass of sample. For example, it is possible to determine the number of aerobic heterotrophic bacteria and yeast in the water, soil, air etc. The platings are done in several parallel replicates (at least 3), using either spread-plate, pour-plate or drop-plate techniques. The inoculum size of the sample or its dilution is then mostly 0.1, 0.5-1.0 or 0.01 ml, respectively. To find the optimal number of colonies, several dilutions need to be plated. Not all viable microbes can be counted this way as there is no universal medium ensuring the growth of all microbes and not all microbes are culturable. Only 0.1-10% of bacteria are thought to be culturable. As the number of colonies grown depends greatly on the medium composition, microbiological works always refer to the medium used. The method is relatively time consuming as it takes 1 to 7 days on average for the formation of colonies, in

case of drop-plate 24-48 h. To compare data, the medium, incubation time and temperature need to be specified in a method.

Colonies are counted either by eye or using electronic colony counters (e.g. Quebec). Counted colonies are marked to prevent recounting the same colonies and an average number of colonies on a plate are found from replicates. Taking into account the average number of colonies, inoculation size and dilution factor, number of viable cells in the sample can be calculated (unit is CFU/ml or CFU/g; see also chapter III).

Koch's plate exposure method is used to determine the number of **airborne** aerobic heterotrophic bacteria. Petri dishes with solid agar media are opened for at least 5 minutes, then covered with the lid and incubated for 3-7 days according to the standard method. The colonies grown from the microbes deposited onto the plate are counted. The microbial number is derived from the postulate stating that **the same number of bacteria found in 10 dm^3 still air settle to 100 cm^2 area in 5 minutes**. The number of aerobic heterotrophic airborne bacteria is expressed as CFU-s per 1 m^3 air.

1.2. Membrane filtration technique

A modified version of plate count method is membrane filtration method that enables to significantly increase the sample volume and thus use this technique for low cell number samples. The sample is not used for direct inoculation, but concentrated by filtering through a sterile 0.2-0.45 µm pore size filter. Then the filter is placed cell side up on a suitable medium plate. As the nutrients diffuse through the filter, the bacterial cells grow into colonies.

Membrane filtration method is often used in sanitary microbiology. For example, the standard method of determining the number of coliform bacteria (foremost *E. coli*) indicating fecal pollution is membrane filtration method (see chapter VI), where the filter is usually put on a selective or differential medium. The most important advantages of membrane filtration method are possibilities to use different media and test large sample volumes in relatively little time. At the same time, the method is often unsuitable if: 1) high amount of particulate matter (turbid water) is present in natural samples, which limits the amount of water that can be filtered; 2) there is high number of background microbes; 3) compounds toxic to bacteria (phenols, metal ions etc.) adsorb onto the filter.

1.3. Most probable number

The most probable number (MPN) method was first introduced by McCrady in 1915 and it is applicable for determining the number of microbial groups from liquid samples. The theoretical basis of this statistical method presumes uniform distribution of microbes in the whole sample and it must be possible to register the existence of a single viable cell according to some specific attributes of its growth (e.g. turbid, formation of gas, pH changes etc.). The method is based on preparing decimal dilution series of the sample, so that the last dilution does not contain microbes of interest (no changes occur in the medium). The number of dilutions depends on the number of microbes in the sample. For example, if inoculation with 1 ml of 10^{-3} dilution gives growth in a medium, whereas 10^{-4} dilution does not, we can say that the MPN/ml is higher than $1x10^3$ and lower than $1x10^4$. To increase the statistical power of the test, each dilution is replicated (at least 3 dilutions in 3, 5 or 10 parallels are done in a standard MPN test). The incubation time and temperature depend on the microbial group of interest. The number of test tubes corresponding to each dilution with positive results (occurrence of growth indicators specific to a certain microbial group) is recorded after incubation and a three digits **MPN index** is composed, where the first digit shows the number of positive test tubes at the highest dilution where highest number of test tubes still show growth. The two following digits show the number of positive test tubes in the next dilutions. If more positive test tubes occur after those two, their number is added to the last number of the index. The microbial number of MPN/100 ml is found from the statistical table (see Table 3) based on Poisson distribution according to the MPN index.

Table 3. Most probable number (MPN/100 ml) determination from multiple tube test (3 of 10-, 1- and 0.1ml inocula) by using MPN index (positive reactions)

Number of positive test tubes in corresponding dilution			MPN/100 ml	95% confidence intervals		Number of positive test tubes in corresponding dilution			MPN/100 ml	95% confidence intervals	
10 ml	1 ml	0.1 ml				10 ml	1 ml	0.1ml			
0	0	0	**<3.0**	–	9.5	2	2	0	**21**	4.5	42
0	0	1	**3.0**	0.15	9.6	2	2	1	**28**	8.7	94
0	1	0	**3.0**	0.15	11	2	2	2	**35**	8.7	94
0	1	1	**6.1**	1.2	18	2	3	0	**29**	8.7	94
0	2	0	**6.2**	1.2	18	2	3	1	**36**	8.7	94
0	3	0	**9.4**	3.6	38	3	0	0	**23**	4.6	94
1	0	0	**3.6**	0.17	18	3	0	1	**38**	8.7	110
1	0	1	**7.2**	1.3	18	3	0	2	**64**	17	180
1	0	2	**11**	3.6	38	3	1	0	**43**	9	180
1	1	0	**7.4**	1.3	20	3	1	1	**75**	17	200
1	1	1	**11**	3.6	38	3	1	2	**120**	37	420
1	2	0	**11**	3.6	42	3	1	3	**160**	40	420
1	2	1	**15**	4.5	42	3	2	0	**93**	18	420
1	3	0	**16**	4.5	42	3	2	1	**150**	37	420
2	0	0	**9.2**	1.4	38	3	2	2	**210**	40	430
2	0	1	**14**	3.6	42	3	2	3	**290**	90	1000
2	0	2	**20**	4.5	42	3	3	0	**240**	42	1000
2	1	0	**15**	3.7	42	3	3	1	**460**	90	2000
2	1	1	**20**	4.5	42	3	3	2	**1100**	180	4100
2	1	2	**27**	8.7	94	3	3	3	**>1100**	420	–

Table is taken from web page of FDA:
http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm109656.htm

This number shows how many microbial cells are probably in 100 ml of the sample or its dilution where the second digit of the index was derived. If MPN index is composed by making dilutions, the dilution factor has to be taken into account and this is seen in the **index middle number**. Let's illustrate finding MPN index and microbial number (MPN/ml) with two examples, a and b with inoculum size 1 ml (using Table 3):

Probe	Positive test tubes in dilution						**Indeks**	**MPN/ml**
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}		
a	3	3	3	**2**	0	0	3**2**0	$9.3x10^2$
b	3	3	**2**	2	1	0	3**2**3	$2.9x10^2$

Most probable number method is widely used in applied microbiology studies (see chapter VI) and it has several advantages: it is simple, allows to determine selectively the number of many microbial groups and the results are reproducible. A disadvantage of this method is that it is laborious as quite a large number of dilutions with several replicates are needed from the sample, and it gives approximate results of number of microbial groups with 95% statistical probability.

In a practical work it is possible to determine the number of **denitrifying bacteria** using the most probable number method and defined Hiltay medium (see APPENDIX II). Decimal dilutions of the sample will be made and three replicates of each will be inoculated with inoculum size of 1 ml. After incubation the number of positive test tubes will be registered. Denitrifiers are facultative anaerobes who use nitrate as a terminal electron acceptor in case of oxygen deficiency and in the process nitrate is reduced to gaseous components: dinitrogen oxide (N_2O) and molecular nitrogen (N_2). Thus, the growth of denitrifiers in a medium is verified by the formation of gases. Additional characteristic is alkalization **throughout the whole medium** (the medium turns blue - a pH indicator bromothymol blue turns blue in alkaline environment) as a result of anaerobic respiration (see chapter IV). Thus, test tubes with formation of gas and color change to blue throughout the whole medium layer are considered positive. Then, MPN index is composed and the number of denitrifiers in the initial sample are found (MPN/ml).

Lately, so called miniature MPN method on microtiter plate has been used to determine the probable number of bacteria in communities, as it enables to determine the number of different bacterial groups by varying media and incubation conditions. Inoculations are done with 8-channel pipette which allows to relatively quickly make 8 replicates of 11 different dilutions on one microtiter plate. For example, to find the most probable number of heterotrophs, optical density of the inoculations are measured after 24-48 h with a multiscanner at 540 nm. Positive samples should have optical density difference of 1.5-2 times compared to a negative control (not containing the sample). The number of heterotrophs (MPN/ml) in the initial sample is found by using computer programs (Klee, EPA or Mike Curiale; http://www.i2workout.com/mcuriale/mpn/index.html). This way it is possible to significantly increase the number of dilutions and replicates without increasing workload. Tetrazolium salts can also be used as growth indicators. For example, nitrotetrazolium blue iodine (INT) (final concentration 0.1-0.6 g/l) is reduced to red formazan by active cells as they oxidize their substrates in a medium, and the formed color can be quantified spectrophotometrically at 492 nm. This redox dye directly competes with molecular oxygen as an artificial electron acceptor, thus allowing to evaluate respirometrically active bacteria. INT is used to determine the MPN index of various physiological groups, whereas the dye is added only few hours before the end of the incubation to prevent its toxic effects on microbial growth.

2. DIRECT METHODS FOR DETERMINING THE NUMBER OF LIVE CELLS

2.1. Counting live bacterial cells under fluorescence microscopy

When fluorescence microscopy is used to determine the number of living cells, the cells are first stained with fluorescent dyes or fluorochromes which absorb light with shorter wavelength (excitation) and emit light with longer wavelength. The excitation and emission wavelengths of some more frequently used fluorochromes in fluorescence microscopy are 470/510 nm for acridine orange (**AO**; acridine-3,6-diamine hydrochloride) and 365/390 nm for 4,6-diamidino-2-phenylindole (**DAPI**).

Nucleic acid specific fluorochromes are used to stain bacteria. AO binds to both DNA and RNA, most probably binding to phosphate groups. It is generally accepted that if bound to a single strand nucleic acid AO fluorescence is orange, whereas if it is bound to a double strand nucleic acid its fluorescence is green. Binding of a basic dye AO to a nucleic acid phosphate group can occur with two mechanisms:

1. Electrostatic or polar binding, which is characteristic to a formation of complex with RNA. It is believed that by binding each phosphate group of a nucleotide, the dye molecules can interact with each other resulting in a metachromatic effect and red/orange fluorescence.
2. Intercalation between neighboring base pairs of different strands of a DNA double strand and there is one AO molecule per three base pairs. In these conditions, the dye molecules are far enough from each other to prevent their interactions and this produces orthochromatic (green) fluorescence.

DAPI is a non-intercalating DNA-specific dye, which gives blue or blue-white fluorescence when bound to DNA. Staining with DAPI allows clear distinction between dead organic matter and bacterial cells as unbound or detrite-bound DAPI has yellow fluorescence. Although DAPI can stain polyphosphate, it preferentially binds to AT-rich (at least three AT pairs) DNA regions. Unbound DAPI gives yellow fluorescence and thus this strong background fluorescence can significantly disturb microscopy. Combinations of several dyes are also used, for example AO together with DAPI, ensuring better contrast compared to AO alone and lower background fluorescence compared to DAPI alone. The binding of AO or DAPI to DNA or RNA cannot be considered specific according to many authors, as the intensity of fluorescence depends also on dye concentration. Using standard staining protocols with AO or DAPI for staining bacterial communities does not allow distinction of live and dead cells.

Nowadays, the number of living cells is most commonly determined with direct counting using **LIVE/DEAD *Bac*Light** (Molecular Probes Inc.) kit, which is based on different cytoplasmic membrane permeability of dead and live cells to nucleic acid-specific fluorescent dyes. The staining working solution contains components A and B (mixtures of dyes) and it is prepared by mixing equal volumes of these solutions immediately before use and kept in the dark. Green dye (**SYTO 9**, for anhydrous compound is ~400 Da) is capable of entering intact cells, while red dye (**propidium iodide**, molecular weight for anhydrous compound is 668 Da) can only enter cells with disrupted membranes. Excitation and emission wavelengths are for SYTO 9 and propidium iodide 480/500 nm and 490/635 nm, respectively. In fluorescent microscopy, the green cells are counted live and red cells dead.

For quantification, a known volume of appropriate sample dilution is filtered through fluorescence-free irgalan black-stained polycarbonate filter (Nuclepore) with pore size 0.2 μm. The filter is stained at room temperature in the dark for 20 min. For preparing the mount, the membrane filter is placed on a microscope slide into special oil, another drop of the same oil is placed on the membrane filter and then it is carefully covered with a cover glass. The bacteria are counted with a fluorescence microscopy in oil-immersion system using 1000-times magnification and an objective with the greatest numerical aperture. The cells are

counted either in the whole field of view or in the squares of ocular grids. The latter makes counting easier as it allows counting cells in a smaller area. The fields of view are changed and in addition to counting the cells inside the squares or grids, the cells on their two sides are also counted, keeping in mind to always number the cells on the same two sides. At least 600 cells are counted in total and at least 20 fields of view are checked.

By calculating the area of a square in the grid (S_{square}), area of the filter (S_{filter}), average number of cells on the square (N) and knowing the filtered sample volume (V) and dilution factor, it is possible to find the number of live cells in the initial sample:

$$\boldsymbol{Cell\ number}\ \left(\frac{\boldsymbol{cells}}{\boldsymbol{ml}}\right) = \frac{\boldsymbol{N \times S_{filter} \times dilution\ factor}}{\boldsymbol{S_{square} \times V}}.$$

3. DIRECT METHODS FOR DETERMINING TOTAL CELL NUMBER

The methods for determining the total bacterial number (**TBN**) are universally applicable in different fields of microbiology (microbe ecology, food microbiology etc.). TBN is also often used for the indirect determination of microbial biomass. All available methods have limitations: 1) method does not allow the distinction of live and dead cells; 2) samples containing a lot of extra organic matter could result in a smaller cell numbers than real; 3) the preparations used for counting can not be further analyzed with other techniques.

3.1. Counting in a counting chamber

The determination of direct cell number of a microbial population is carried out using special counting chambers (e.g. Petroff-Hauser chambers and its modifications). Counting chambers are thicker microscope slides which have two different size square grids engraved on their surface: the larger squares have an area of 0.04 mm^2 and the smaller ones 0.0025 mm^2. The grids are in an indent with a height of 0.1 mm and to ensure a uniform thickness of suspension layer, it is covered with a cover glass so that it adheres tightly to the ground surface of the microscope slide and Newton's rings form on the edges (pattern of rainbow colors). To fill the chamber, a drop of suspension is carefully placed near the edge of the cover glass, so that capillary forces draw it to the chamber. Channels are engraved to the chamber to collect and remove excessive suspension. Some counting chambers may also have two chamber systems engraved, thus allowing to study different dilutions of the sample or replications.

Counting chamber is microscoped in a dry system with a 40x magnification. Depending on the cell density, the cells are counted in larger or smaller squares. All cells inside the square and cells on the two sides of the square are counted. It would be statistically correct to have about 10 cells in a smaller chamber (square) and about 30 cells in a bigger one. This prevents overlapping of the cells. Cells are counted in at least 5-10 big squares or 10-15 small squares. An average number of cells in a chamber (N) is found and knowing the volume of counting chambers ($V_{chamber}$, **mm^3**) allows to calculate microbial number in **1 ml of initial sample**. To unify the units, it must be kept in mind that **1 ml = 10^3 mm^3**. If the sample was first diluted, the cell number needs to be multiplied with the dilution factor:

$$\boldsymbol{Initial\ sample\ cell\ number}\ \left(\frac{\boldsymbol{cells}}{\boldsymbol{ml}}\right) = \frac{\boldsymbol{N \times dilution\ factor}}{\boldsymbol{V_{chamber}}}.$$

The method is simple and low-cost and gives information about bacterial cell shape and size. The limiting factor of this method is a low number of microbes (fewer than 1 million cells) in a sample as the sample volume is small. The counting is definitely disturbed by motility of

the bacteria and to some extent also aggregated cells. Thus, the samples are thermally treated or chemically fixed with formaldehyde (HCHO) to inactivate bacterial movement.

3.2. Counting on membrane filters by light microscopy

Membrane filter method has been used since the beginning of 1930s. The method is based on filtering the cells on a sterile membrane filter and staining them with a basic dye carbol erythrosine. Filtration takes place in a vacuum system using previously sterilized Zeitz support stand. Sterile filter papers are placed below a membrane filter with pore size 0.2 µm (glossy surface up) for uniform distribution of the vacuum. The volume of the sample to be filtered is usually 3-10 ml. The filter with cells is stained on a filter paper disc immersed with carbol erythrosine on a Petri dish for 3-5 hours. To reduce the background color of the filter, it is washed with distilled water on wet filter paper discs, changing the latter often. The filter needs to remain light pink after washing. The filters are dried in air and stored in dark until microscopy. Part of the filter is used for making the mount by placing it cell-side up to a drop of immersion oil on the microscopy slide, so the membrane filter turns translucent. Another drop of oil is then added and the cells are counted using oil-immersion objective. The ocular is equipped with a reticle for easing the counting. At least 15-20 different fields of view or squares with the same placement in different fields of view are counted. The average number (N) of bacteria in a square or field of view is found and by calculating the filtering area of the membrane filter (S_{filter}), area of the square or field of view (S_{square}) and taking into account the sample volume (V) and if needed a dilution factor, the bacterial number in 1 ml initial sample can be found (see also chapter 2.1)

3.3. Counting on polycarbonate filters by fluorescence microscopy

When previously mentioned methods for determining GBN are currently only used in laboratories without access to fluorescence microscope, nowadays the best method for determining TBN is staining cells with fluorochromes and counting under epifluorescence microscope. The earliest works in this field date back to 1940, when this method was applied for determining the number of soil microbes. This method enabled to discover that the bacterial number could be even several orders of magnitude higher compared to plate count method. In addition to a standard method of staining with AO or DAPI, **LIVE/DEAD *Bac*Light** (Molecular Probes Inc.) kit can distinguish between live and dead cells, as described earlier.

Fixed natural samples stored at 4 °C can be used for determining total microbial number. Preparation is obtained by fixing 2-10 ml of suitable dilution of the sample on a 0.2 µm pore diameter membrane filter. Both irgalan black stained polycarbonate (Nuclepore) membrane filters and aluminum oxide filters (Anopore) are used, whereas the latter gives 20-30% higher numbers. To ensure more uniform distribution of the bacteria, the sample should be dispersed prior filtration using sonicator, homogenizator or Vortex. The filtration is carried out in a vacuum system. For uniform distribution of the pressure, another filter with pore size of 0.8-1.0 µm is placed below the membrane filter. Staining with AO takes place at least 3 minutes in the filtration system. The dye can be added to the sample before the filtration or to the bacteria on the filter after filtration. After staining, the filter is washed with sterile water to decolorize the background. Then a preparation is made for microscopy, just as in counting live cells (see chapter 2.1). Filters stained with AO stay unchanged for at least a week, those stained with DAPI up to 24 weeks if stored in dark at 4 °C. If the sample is stained with AO, cells with both green and orange fluorescence can be seen in the preparation. Despite the fact that the green and orange fluorescence in the preparation is associated with a physiological state of the microorganism, it is not acceptable to distinguish live and dead cells only by the color.

3.4. Electronic counting

For a routine counting of bacteria, electronic counting with Coulter counter is used. The method is based on the principle that small poorly conductive particles like bacteria cause change in impedance when passing through an orifice between small electrodes. As a particle passes through the orifice, it displaces a certain volume of electrolyte solution in a sensitive zone, thus causing a pulse in impedance which is registered. Precise and reproducible results can be obtained by regulating sample flow rate. The registered current changes depend on the particle size, whereas the microbial cell size is very close to the minimal limit. The limitation of this method is not only the high price of the counter, but also that bacteria cannot be distinguished from other particles in the sample. Thus it cannot be used for analyzing natural samples.

B. BIOMASS DETERMINATION

Bacterial growth does not only comprise cell division but also increase in cell mass which is related to the synthesis of cell material. The growth is balanced if increase in biomass is proportional to the cell number. This is not the case in lag phase and late growth phases. In lag-phase, the cell biomass increases without increasing number of cells, whereas in the end of log phase, cell size is reducing and cell number increases more compared to biomass. For this reason, these two parameters describing microbial populations have to be distinguished.

1. 1. DIRECT METHODS

1.1. Determining biomass through weighing

This is the most straightforward and simple method, although also the most time-consuming. Samples are drawn from the culture after certain periods of time, centrifuged at maximum speed to separate cells from the centrifugate or filtered through membrane or paper filter. Biomass is determined either as wet or dry weight. Collected cells are washed and weighed in a pre-weighed container (pre-weighed together with filtering equipment). For dry matter determination, the tare weight of absolutely dry container and filtering equipment is taken and then the weight together with dry biomass is taken. Microwave oven or thermostat (100-105 °C) can be used for drying the matter to its dry weight. Dry matter (**DM**) content can be found from the following equation:

$$DM\ (mg/ml) = (A-B) / V,$$

where A is the laden weight with cells (mg), B the tare weight (mg) and V – the volume of the suspension (ml).

Weighing can give different results depending on the growth conditions of the microbe. Thus, microbes producing exogenous polysaccharides give higher dry weight if sugar concentrations in the media are higher. The limitation of this method is need for rather high volume of the centrifuged or filtered sample. At the same time it is the only method for evaluating the growth of species that have filamentous or flocculent growth.

2. INDIRECT METHODS

2.1. Turbidity of cell suspension

The most commonly used biomass determination method in laboratory practice is measuring cell suspension turbidity with spectrophotometry, which allows relatively quick and precise estimation of microbial biomass. The method is based on measuring the absorption of light by the cells, whereas the intensity of absorbed light is proportional to the cell number. According to the Lambert-Beer law, the attenuation of light transmitted through a medium is related

exponentially to the absorption coefficient (extinction coefficient), solution concentration and the optical path length (solution thickness). In standard conditions, the extinction (absorption) is called optical density (**OD**), whereas the numerical value of the subscript corresponds to the wavelength used for the measurement. The wavelength of the light must be chosen to ensure maximal absorption. The turbidity of microbial suspensions is measured at 420-600 nm. This higher wavelength light is used because the cells absorb it the best and the medium color (often yellow) is interfering the least. This law is only applicable if the particles (cells) do not disturb each other absorbing the light that is at low concentrations. To minimize the error, the high density suspension needs to be diluted. Most spectrophotometers have two scales: logarithmic OD scale and arithmetic transmission (T) scale. Optical transmission is the ratio of the intensity of transmitted light (I) to the intensity of incident light (I_0) and $OD=-logT$.

The OD values do not provide us knowledge about cell number and thus it is rational to construct a calibration graph describing the relationship between the cell biomass and cell number, which is strictly species and even strain specific. Different dilutions are made for constructing a calibration curve for determining the cell number and if necessary, OD. Depending on the method used for determining the cell number, the calibration curve illustrates the relationship between the optical densities of a specific microbial population and either it's live or total cell number. The samples need to be kept on ice to prevent cell division and in case of motile bacteria, also fixed with 1% formaldehyde. The method is not applicable to microbial suspensions with very low cell number. The cell number has to be at least 10^7 cells/ml, so that the turbidity can be measured.

2.2. Amount of cell components

Biochemical analyses are used more often for biomass determination if the sample has low cell number. The amount of various microbial cell components is measured (e.g. total protein, DNA, specific genes, enzymes, RNA etc.). In general, determination of the amount of a single cell component is laborious and can vary depending on the growth phase. For example, cells in active growth phase contain more DNA per cell compared to cells in stationary phase.

QUESTIONS

1. Why is the cell number not expressed in cells/ml in plate count method?
2. Which inoculation methods are used if the cell number is determined with plate count method?
3. Which microbial groups number is determined with nutrient agar spread plate?
4. Find the CFU/ml in the initial sample, if 290 colonies grew on 20 ml of nutrient agar when 100 µl of a dilution (1 ml of the culture is transferred to 9 ml of water, 0.1 ml of the dilution obtained is transferred to 9.9 ml of water) was plated?
5. Why are the bacterial cell numbers different when determined with direct counting by microscopy and using a plating method? Which method is more precise?
6. Why is it necessary to fix microbiological samples and which fixing agents are used?
7. Is membrane filtration technique used with direct or indirect determination of bacteria?
8. Name advantages and disadvantages of a membrane filtration method?
9. How is MPN index determined with the most probable number method?
10. How many dilutions should be made for the MPN method?
11. What is the criterion for distinguishing live and dead cells with LIVE/DEAD kit?
12. What problem could arise with distinguishing live and dead cells with LIVE/DEAD kit?

13. Is it possible to determine the total number of microbes in drinking water using counting chamber? Explain.
14. What are the most common methods for determining the biomass?
15. What method would you use for determining the biomass of a microbial suspension with a flaky consistency?
16. What is spectrophotometric determination of biomass based on?
17. Are live or dead cells measured with spectrophotometry?
18. What is optical density (OD)?
19. What method would you choose for determining biomass if the cell number is very low?
20. What should you do if you want to determine live and total cell number based on OD values?

PRACTICAL WORKS AND RESULTS

A. DETERMINATION OF BACTERIAL NUMBERS

Sample: natural water (works no 1-4 and 6). All calculations should be carried over to the table at the end of part A.

1. Determination of live cell number with indirect methods

Work no. 1. PLATE COUNT METHOD - SPREAD PLATE – determining the number of heterotrophs

1) **Each student** has to make a decimal dilution series (10^0-10^{-4}) of the sample water into 1.5 ml eppendorfs (see chapter III), keeping in mind to **shake** the initial sample and intermediate dilutions and **replace the tips**. Mark 2 nutrient agar (**NA**) plates, make spread plates (inoculation volume of 0.1 ml) using the dilutions suggested by the supervisor and incubate them (dish bottoms up) at room temperature.
2) After 7 days, count the colonies, calculate the number of heterotrophs in the initial sample (CFU/ml), find the average value of the inoculations and fill out the table (p. 64).

Work no. 2. PLATE COUNT METHOD – DROP-PLATE – determining the number of heterotrophs

1) Divide the **bottom** of a NA plate into four sectors and mark these according to the dilutions being plated (10^0-10^{-3});
2) into each sector, plate three drops of a dilution (**from the dilution series made into 1.5 ml eppendorfs**) with inoculum size of 10 µl according to the markings. The agar must not be punctured with a pipette tip!
3) Let the inoculum absorb into the medium for 10-20 min (**do not shake or turn the dish around**);
4) incubate the dishes at room temperature for 24-48 h (dish bottoms up);
5) calculate the number of heterotrophs in the initial sample (CFU/ml) for each dilution, find the average and fill the table.

Work no. 3. PLATE COUNT METHOD – FILTERING METHOD – determining the number of *E. coli*

1) **Per 5 students** make a decimal dilution series (10^0-10^{-5}) with the final volume of 50 ml into sterile 100 ml Erlenmeyer flasks. Keep in mind to **shake** the initial solution and each intermediate dilution (0.5-1 min). Use a new pipette tip for making each new dilution!
2) **Each student** filters 1-5 ml of **one** dilution (**from the dilution series made into 100 ml Erlenmeyer flasks**) suggested by the supervisor through a sterile membrane filter (pore size 0.2 µm) and places the filter, cells up, on the **Endo agar**. Make sure that the filter is in tight contact with the agar, meaning that no air bubbles are between the filter and agar. **NB! Three filters can be fitted on one Endo agar plate!**
3) Incubate the dishes at 37 °C for 24-48 hours (dish bottoms up);
4) count the colonies of *E. coli* (dark red, dry, with a golden green metallic sheen, see chapter II);
5) calculate the number of *E. coli* in the initial sample (CFU/100 ml), taking into account both the dilution and volume of sample gone through the filter;
6) fill the table in the end of part A with the results (p. 64).

The **filtering** goes as follows:

- work in the vicinity of flame;

- take the drum of the filtering equipment between the tweezers and dip the bottom part with the thread into ethanol in a Petri dish, shake off excessive liquid and ignite the ethanol left on the drum. When the ethanol has burned, place the drum, thread on the top, on the table to cool (near the flame);
- place the lower part of the filtering equipment (filter holder) on a vacuum flask (Bunsen flask) and connect it with a vacuum source (a water pump);
- with sterile tweezers, place 2 sterile paper filters and a sterile membrane filter on the filter base;
- turn the cooled sterile drum on the filter base. Before filtration make sure that the drum is properly screwed on!
- Measure and add a certain volume of sample on the filter and generate vacuum by turning on the faucet;
- when the filter is dry, first let the air in the Bunsen flask by unscrewing drum of the filtering equipment from the base and then turn off the faucet;
- in the vicinity of the flame, open a Petri dish with Endo medium and using sterile tweezers, place the filter on the dish, as described earlier. Mark the bottom of the dish.

Work no. 4. MOST PROBABLE NUMBER METHOD (in test tubes) – determining the number of denitrifying bacteria

To determine the number of **denitrifying bacteria** make **1 replicate for the same sample within one group**.

1) Mark test tubes containing Hiltay medium with dilutions of 10^0-10^4 in three replicates;
2) inoculate each test tube with 1 ml of sample from the **dilution series** made into **100 ml** Erlenmeyer flasks (see previous work). After adding the sample do not shake the test tubes!
3) Incubate these test tubes for 7 days at room temperature;
4) determine the number of test tubes with positive results (gas bubbles, pH change throughout the whole medium) and compose an MPN index. To find the number of denitrifiers use the Table 3 above (chapter V 1.3) or respective computer program;
5) fill the table at the end of part A with the obtained results - number of denitrifying bacteria in the initial sample (MPN/ml).

Work no. 5. KOCH'S PLATE EXPOSURE METHOD

1) Keep a Petri dish with **nutrient agar** open for longer than 5 min. Choose different places for the exposure. Each students makes one such plate. Mark the lids of the plates (name, exposure place and time) and put the plates (bottom up) in an incubator at room temperature.
2) Calculate the number of bacteria in air (CFU/m^3) according to the postulate presented in the theoretical part (chapter V A 1.1):

CFU/m^3 in air =

2. Determining the number of live and total bacterial cells with a direct method

Work no. 6. DETERMINING THE NUMBER OF LIVE CELLS WITH A DIRECT LIVE/DEAD *Bac*Light METHOD

1) Carry out the filtration near the flame;
2) place the base of the filtering equipment with a glass filter washed in ethanol on a Bunsen flask, then put there a membrane filter with a large pore size and then a polycarbonate filter treated with irgalan black (pore size 0.22 μm);
3) place the drum of the filtering equipment on the filter and clamp it on the base;
4) add **1 ml** of a thoroughly vortexed sample on the filter, filter it dry and wash the filter with 1-2 ml of sterile distilled water;
5) when the filter is completely dry, let the air into the filtering equipment through the cap of the Bunsen flask and turn off the faucet;
6) mix the working solutions of SYTO 9 and propidium iodide dyes from the LIVE/DEAD *Bac*Light kit in the ratio of 1:2 (total volume of **3 μl**), dilute the mix to **1 ml** with sterile distilled water, mix on the Vortex and pipette it carefully on the filter down the wall of the drum;
7) let the filter stain for 15-20 min (in the dark);
8) filter the dye mixture and make sure that no water is left on the filter, then turn off the faucet. Then make the preparation: place 1 drop of *Bac*Light oil on the microscope slide, place the filter on it and add another drop of *Bac*Light oil on the filter and cover it with a cover glass. Excessive oil can be removed with an adsorbing paper by carefully pressing the cover glass;
9) examine the slide under fluorescent microscopy in immersion system using fluorescence-free immersion oil;
10) count the live (green fluorescence) and dead (red fluorescence) cells in a grid with a certain area.
    Find the **live and total number of the bacteria** (cells/ml) in the initial sample by taking into account the average number of cells, filter area, the area of the grid used for counting, sample volume and dilution. Fill the results in the table at the end of part A (p. 64).

SUMMARY

Fill the table with the results that you have obtained during the analysis of natural water (practical works from chapters V and VI), include the data of the second sample analysed by your groupmade. Under the table, write the conclusions made after comparing the results of two samples, consider the role of applied methods.

Method	**Physiological group**	**Unit**	**Sample I**	**Sample II**
Plate count method – spread plate (V; work no. 1)	Heterotrophs	CFU/ml		
Plate count method – drop plate (V; Work no. 2)	Heterotrophs	CFU/ml		
Plate count method – filtering method (V; Work no. 3)	*E. coli*	CFU/100 ml		
Most probable number method (V; Work no. 4)	Denitrifying bacteria	MPN/ml		
Most probable number method BGLB broth (VI; Work no.1)	Coliform bacteria	MPN/100 ml		
LIVE/DEAD total number of bacteria (V; Work no.6)	Total number of bacteria	cell/ml		
LIVE/DEAD live number of bacteria (V; Work no.6)	Live number of bacteria	cell/ml		

B. DETERMINING THE NUMBER WITH A COUNTING CHAMBER AND BIOMASS WITH SPECTROPHOTOMETRY

Work no. 7. Determining the relationship between *E. coli* wt (wild type) **cell number and biomass**

Sample material: Different dilutions of an overnight culture of *E. coli* wt.

1) Mix the control solution carefully on Vortex;
2) measure the optical density of the microbial suspension (λ=580 nm, OD_{580}) with a spectrophotometer and decide based on that:
   a) If OD is in the range of 0.1-0.8, continue with step 3;
   b) If OD is higher than 0.8, the sample must be diluted in order to measure precise optical density. Don't forget to mix the sample when making dilutions!
3) In order to use counting chamber the solution must have OD in the range of 0.1-0.15. If the optical density of the sample is higher, it must be diluted. Don't forget to mix the sample when making dilutions!
4) Transfer the sample carefully to Gorjajev's counting chamber (see theoretical part in chapter V point 3.1). NB! Mix the suspension on Vortex carefully before filling the chamber!
5) Count the cells in and on two edges of 15 small squares;
6) calculate the average cell number of the sample (cells/ml), taking into account the volume of the counting square, sample dilution and average number of cells;
7) insert the optical density of the analyzed sample into an Excel file created by the supervisor; construct a calibration curve using the data of the whole group, so precise initial sample OD_{580} is put on the y-axis and total cell number in your studied sample on the x-axis. Add a linear trend line equation.

1.	6.	11.
2.	7.	12.
3.	8.	13.
4.	9.	14.
5.	10.	15.

Initial sample OD_{580} =
Dilution used for measuring optical density =
Diluted initial sample OD_{580} = Precise initial sample OD_{580} =
Used dilution with suitable optical density for the counting chamber =

Average cell number (cells/ml) in the study sample =

VI Microbiological analysis of water

It is important to test both drinking water and water where people use to swim in for pathogenic microbes capable of harming humans. Microbes originating from gastrointestinal tract are not very resilient in outside environment and being in physiological stress they lose quickly the ability to form colonies in differential- and selective media. In outside environment their viability is lowered by temperature, sunlight, effects from local bacterial population and the chemical composition of the water. For these reasons coliform bacteria are used as indicator organisms for fecal contamination of water. Among them are inhabitants of both humans' and other warm blooded animals' gastrointestinal tracts who do not reproduce in water but do keep their viability for longer time than several of the pathogens that might also happen to be carried to the same environment (*Salmonella, Shigella, Leptospira, Vibrio*). The detection of coliform bacteria is also done by simpler laboratory methods. In the following section we get to know some methods of evaluating the quality of water samples.

A. DETECTING COLIFORM BACTERIA

Coliform bacteria (*Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes, Citrobacter freundii*) belong to enterobacteria and it is characteristic to all of them to **ferment lactose at high temperature forming acid and gas in 48 hours.** Although all coliform bacteria are not the residents of gastrointestinal tracts their abundance is used as an index for fecal contamination of water – amount of coliform bacteria in 100 ml of sample. In case this number is zero and the general amount of heterotrophs in sample does not exceed 100 cells per ml it is generally safe to drink such water. Out from all coliform bacteria some 60%-90% are fecal bacteria while out of these 90% are *E. coli*. This is one reason that *E. coli* as an inhabitant of warm blooded organisms' gastrointestinal tract is fit for use as an indicator microbe of fecal contamination. In addition to that, it is easy to test microbiologically and its resistance to outside environment is better than that of pathogens. To separate the inhabitants of gastrointestinal tract (*E. coli*) from those unrelated (*E. aerogenes*) several additional tests are performed.

Classically the existence of coliform bacteria in a sample is confirmed by presumptive test, confirmed test and completed test – where each of these tests is based on one or more characteristic of coliform bacteria.

Presumptive test consists of incubating the sample (24 h, 37 °C) inside lactose containing medium equipped with gas tubes (Durham tubes). Contrary to other enterobacteria the coliform bacteria ferment lactose and the resulting gas pushing the medium out of the Durham tubes presents a sign of coliform presence.

Confirmed test is used to confirm the existence of coliform bacteria from those samples testing positive in presumptive test, or exhibiting growth and pH changes but emitting little gas. Usually, a plating is done onto selective-differential medium (media) for example Endo agar plate, which only allows GN bacteria to grow and thus differentiates coliform bacteria from non-coliform.

Completed test is done to isolate those pure cultures acquired by confirming test doing substantive tests to identify coliform bacteria and especially *E.coli* (staining by Gram, the formation of acid and gas inside lactose containing broth).

As an alternative one can combine the tests and add more contemporary tests to detect *E.coli.*

In this practicum we use most probable number method from serial dilutions using 2% Brilliant Green Lactose Bile broth 2% (**BGLB**) to determine the abundance of coliform bacteria (presumptive test). BGLB will be made with different concentrations depending on

the amount of inoculum. The broth is selective thanks to brilliant green and bile salts, as the first inhibits GP bacteria (including lactose fermenting GP bacteria) and the second inhibits non-coliform GN bacteria and those that live outside of gastrointestinal tract. Lactose is the substrate of fermentation, which helps to differentiate coliform bacteria (when incubating at 37 °C for 48 h) by **producing gas** (broth is pressed out from Durham tubes) and by **coloring the indicator dye due to the formation of acidic environment.** Based on acquired results their likely abundance is found in fermentation test (MPN/100 ml in original sample, refer to chapter V, paragraph 1.3.).

It is important to know different fermentation types of enterobacteria for the tests to determine coliform bacteria. Namely, *E. coli* and *Citrobacter* belong to the type performing **mixed acid fermentation** where alongside acid and neutral fermentation products (4:1 ratios) the mixture of CO_2 and hydrogen is produced (in ratios CO_2:H_2; 1:1). In the case of **butanediol fermentation** (chapter VIII, for example *Enterobacter* and *Klebsiella*) CO_2 dominates in gas mixture (CO_2:H_2; 5:1) while the ratio between acidic and neutral products is 1:6, respectively. From this it can be seen that in the case of mixed acid fermentation the production of gas is severely shifted to H_2 which as a gas dissolves badly in water and is easily detected in the gas tubes. On the other hand, CO_2 as an easily soluble gas (in water) is quickly removed from the environment. The production of H_2 starts from **formate,** which is transformed to H_2 and CO_2 by formate hydrogenlyase and specifically in the case of mixed acid fermentation the buildup of formate is more intense.

The following "confirmed" or "completed" tests can be used to confirm the presence of *E. coli* in the samples that have positive results in presumptive test or samples that had growth and changes in pH but little emission of gas:

1) Inoculation into LB broth and after overnight incubation performing **indole test** (chapter VIII). Positive result for converting tryptophan to indole confirms the presence of *E. coli.*

2) Inoculation to selective-differentiating medium (for example Endo medium), where the relatively dry colonies of *E. coli* acquire dark red color and greenish-metallic luster, *Enterobacter* forms red colonies on Endo medium and non-lactose-fermenting GN strains form colorless colonies with slimy consistency. By the characteristics of grown single colonies the abundance of coliform bacteria and *E.coli* is determined using the **membrane filter technique** (chapter V, paragraph 1.2.).

3) Nowadays several supplementary "completed" tests are quite commonly used for the detection of *E. coli* that are based on its unique feature of producing **β-D-glucuronidase (GUR)**. Namely, 97% of non-pathogenic *E. coli* strains have the gene *gusA* coding for the required enzyme, while the other coliform bacteria do not. β-glucuronides are growth substrates in the case the bacteria have inducing permease and GUR and the resulting product is D-glucuronate. Several commercial +/- tests promise to test for coliform bacteria and *E. coli* in the span of 24 hours or even less. These tests are based on the presence of **β-galactosidase** (which breaks β-1,4-glucosidic bonds and is common to all coliforms) and β-D-glucuronidase. The results are achieved by both chromogenic and fluorogenic substrates that are added to the growth medium. For example the **Colilert test**, where β-galactosidase is detected by adding chromogenic substrate ***ortho*-Nitrophenyl-β-galactoside** (**ONPG**) and the β-glucuronidase is detected by adding fluorogenic substrate (4-methylumbelliferyl-β-D-glucuronide, MUG) as single source of C. When the coliform bacteria are present in the sample the medium will turn yellow as the colorless OPNG hydrolyses releasing o-nitrophenol (refer to chapter VIII). The presence of *E. coli* in the same sample is indicated by the fluorescence of the medium under the longer wave-lengths of UV light (365 nm). Fluorescence is caused by 4-methylumbelliferyl released from MUG by the action of GUR. In addition to the substrates mentioned before β-galactosidase can be detected by fluorogenic 4-methylumbelliferyl-β-D-galactopyranoside (MUGal) and β-D-glucuronidase by chromogenic 5-bromine-4-chlorine-3-indolyl-β-D-glucuronide (X-gluc). On agar plate we get for the first case positive colonies as fluorescent thanks to the production of methylumbelliferyl from the

hydrolysis of MUGal and on the other case we get positive colonies as blue thanks to the release of bromo-chloro-indigo from X-gluc.
4) To determine *E. coli* by genotypic features the target is **GUR coding gene *gusA***. This gene is 1808 base pairs (bp) long and is part of the *gusRABC* operon. During the polymerase chain reaction (PCR) a 623 bp long fragment of it is amplified.

B. POLYMERASE CHAIN REACTION (PCR)

PCR (*polymerase chain reaction*) is one of the main methods of current molecular microbiology. This method allows to amplify specific, usually up to 10000 bp long regions of DNA in *vitro* by using enzymatic replication performed by DNA-polymerase.
To perform PCR following components need to be mixed together:
1. **Target-DNA**, which contains region to be amplified. For this, already isolated and cleaned DNA is suitable as well as bacterial cells which lyze during PCR and whose DNA being released would then be the actual target. The amount of target-DNA is usually within range of 0.01-1 ng for plasmidic or phage DNA and 0.1-1 µg for chromosomal DNA;
2. **2 primers** (as a rule) – short, usually 15-30 bp long one stranded pieces of DNA, which are complementary to the 3' ends of the double stranded DNA being amplified in such way that polymerase starting from the 3' end replicates the region of amplification (Fig. 1). To design primers one needs to know the nucleotide sequence for the strand ends of the region to be amplified;
3. **thermostable DNA-polymerase**, which has the temperature optimum of about 70-74 °C. Most often the polymerase *Taq* isolated from a bacteria from hotwater springs *Thermus aquaticus* (optimum of 72 °C);
4. **deoxyribonucleotides** (dNTP), out of which the new strands will be synthesized;
5. **buffer**, which will guarantee optimal chemical conditions for DNA-polymerase activity and stability;
6. **2-valence cations,** usually Mg^{2+}, which work as co-factors for DNA-polymerase.

PCR reaction is performed inside special thin walled tubes using cycling thermostat and 20-35 cycles – whereas each cycle is composed of three stages (Fig 1):
1. **the denaturation of DNA** at high temperature (94-98 °C) 20-30 seconds (there comes the need for thermostable DNA-polymerase!);
2. **the annealing of primers** (20-40 seconds), the temperature needed for primer annealing T_S (usually in the range of 50-70 °C, as similar as possible for both primers) is chosen dependent on primer length and nucleotide composition using the simplified equation:

$$T_s = [4(G + C) + 2(A + T)] - 5\mathbf{;}$$

3. DNA-polymerase attaches to small double stranded DNA regions created by annealed primers and after the temperature is raised to 72 °C the **DNA synthesis** starts. The duration of this step is dependent on the synthesizing speed of the polymerase. Usually it is 1000 bp per minute.

As a rule, a so-called **initiation** stage is performed before these cycles, where denaturation of DNA at 94-98 °C for 1-9 minutes guarantees the state where most of the target DNA and primers are single stranded. Also it is common to add a so-called **extension** stage after the cycles (5-15 min) during which the polymerase can finish synthesizing all the strands still in progress. The presence, approximate concentration and/or size of product(s) acquired through PCR is usually checked by the means of agarose gel electrophoresis.

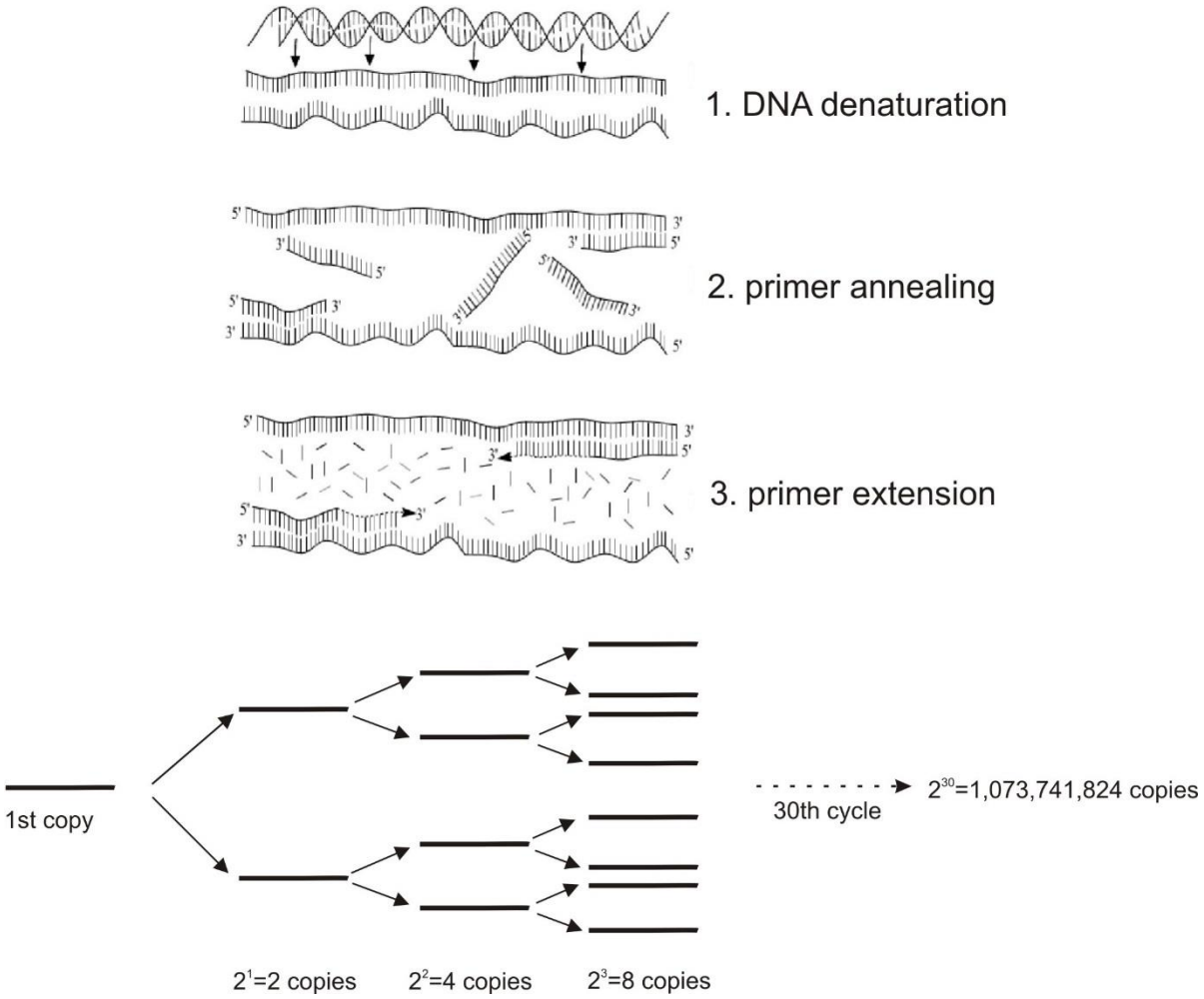


Figure 1. General schema of PCR.

C. GEL ELECTROPHORESIS

Gel electrophoresis is a method to separate RNA, DNA and protein molecules by their size using the motive force of an electrical field. Usually some kind of cross-linked polymer is used as the matrix of the gel. The resolution of the method depends on the chosen matrix and its concentration. The smaller the differences in size of the molecules we want to separate the denser the matrix needs to be. To separate proteins and smaller nucleic acids mainly polyacrylamide is used, starting from the nucleic acids that are about 50 bp long agarose is used. Gels are usually produced with the use of TAE (Tris-acetate-EDTA) or TBE (Tris-borate-EDTA) as the buffer, having the pH of ~8. TAE buffer has smaller buffering capabilities, but produces better resolution for larger molecules.

100-50 000 bp DNA fragments are separated on gel which has agarose concentration of 0.3-2%. As the DNA molecules have negative charge at pH~8, they will move towards the anode when electric field is applied to the gel, with smaller molecules moving faster through the gel. In addition to the molecular size, their conformation affects the movement – circular molecules will move either slower (in case of open circular molecules) or faster (supercoiled) compared to linear molecules of the same molecular mass/linear length in bp.

Approximately 5 V/cm (cm, denoting the distance between electrodes) guarantees the best resolution. Applying too much voltage can cause the gel to melt which reduces the

resolution. Alternatively one can perform the electrophoresis in an environment with lowered temperature.

To determine the size of molecules being analyzed they are flowed alongside the so called size-marker which is a mixture of molecules with known sizes.

To transfer the DNA samples to a gel a so-called „loading-buffer" is used which contains glycerol. Thanks to this the sample sinks to the bottom of the sample well molded into the gel. To visualize the sample, negatively charged indicator dyes like bromophenol blue or xylencyanol are added – which have approximately the same mobility as 300 or 5000 bp DNA fragments, respectively (also dependent on the agarose concentration).

After completing the electrophoresis the molecules have to be made visible inside the gel. For DNA molecules the UV fluorescent ethidium bromide (EtBr) is used, the molecules of which intercalate between the DNA bases. EtBr can be added already when making the gel at final concentration 0.5 µg/ml. Other fluorescent dyes are also used like SYBR Gold, SYBR Green, Oxazole Yellow etc.

QUESTIONS

1. Name features characteristic to coliform bacteria.
2. Why is filtering contaminated water through a membrane filter not enough to use it as a drinking water?
3. Why is it important to use indicator organisms to evaluate the water quality?
4. What important characteristics of *E. coli* allow it to be used as an indicator organism?
5. What methods are used to test for the abundance of coliform bacteria?
6. Why are the abundances of coliform bacteria given as per 100 ml samples not per 1 ml?
7. Can the amount of colonies formed on Endo medium be used as the CFU of the sample? Reason why.
8. What is the importance of presumptive test in the determination of coliform bacteria?
9. What can cause a positive presumptive test to be flagged as negative result in later confirming or additional test?
10. Can some other sugar than lactose be used in the medium meant to test for coliform bacteria?
11. Name the characteristics of BGLB medium that make it selective and differentiating.
12. What is meant by the term coliform index?
13. In which ways *E. coli* differs from other coliform bacteria?
14. Name at least 3 substrates grown on which the *E. coli* will show positive?
15. How to react when testing for *E. coli* the indole test gives positive result but GUR activity is non-existent?
16. Why is it necessary to use two primers when performing PCR?
17. Why is it necessary to use thermostable DNA-polymerase when performing PCR?
18. Why is the gel for gel electrophoresis produced in buffer solution not in regular water?
19. How are DNA fragments separated in gel electrophoresis?
20. Why the size marker is also usually flowed alongside the molecules being investigated when performing gel electrophoresis?
21. What mistake have you made when during the gel electrophoresis the marker moves in the wrong direction?

PRACTICAL WORK AND RESULTS

Material to be studied: the same water sample as per chapter V practical works no. 1-4 and 6.

Work no. 1. Most probable number method to determine the abundance of coliform bacteria

1. As **a group** make inoculations from the water dilutions suggested by the supervisor (the dilutions inside 100 ml Erlenmeyer flasks; look the practical work 3 from the last chapter) into BLGB liquid media containing test tubes equipped with Durham tubes. For each dilution, inoculate three different tubes with inoculum sizes of 0.1 ml, 1 ml and 10 ml. In case of inoculum size of 10 ml take heed that the inoculation is performed into **2 times more concentrated BGLB broth. Before taking the inoculum, shake the samples well!**
2. Incubate in thermostat at 37 °C;
3. find tubes testing positive at 24 and 48 hours for all dilutions and inoculum sizes and fill the results into the following table;
4. according to derived MPN find out the probable amount of coliform bacteria in the studied water. Use Table 3 from chapter V (1.3);
5. for comparison one can also use Thomas equation to find the abundance of coliform bacteria:

$$\boldsymbol{Coliform\ abundance}\left(\frac{\boldsymbol{MPN}}{\boldsymbol{100\ ml}}\right) = \frac{\boldsymbol{positive\ test\ tubes(pcs.)} \times \boldsymbol{100}}{\sqrt{\boldsymbol{volume\ of\ negative\ test\ tubes\ (ml)} \times \boldsymbol{volume\ of\ all\ test\ tubes(ml)}}}$$

Sample	Dilution	Inoculum size (ml)			MPN/100 ml of sample	average MPN/100 ml of sample
		10	1	0.1		

Work no. 2. Testing for GUR activity

In first practicum:

1) make a streak plate from **one positive (with gas bubble) or one negative but with visible growth test tube from work 1** on the R2A agar plate. Definitely mark down the dilution and inoculum size that went into the test tube. Incubate the plate at 37 °C.

In second practicum:

2) take some cells from the plate produced in first practicum and make a streak plate on one sector of sodium lauryl sulfate+MUG plate. As a positive control inoculate the same plate with *E. coli* and as a negative control with *Enterobacter aerogenes*;
3) incubate in thermostat at 37 °C.

In third practicum:

4) observe the SDS+MUG plate under UV and explain the results.

Work no. 3. PCR TO TEST FOR *gusA* GENE

Material to be studied: cells from the R2A plate sector inoculated in previous work.

1) First make a **lysate** from the cells under study. This will later be used as the target DNA in the PCR. For this purpose pipette 50 µl sterile water into PCR tube, suspend a small amount of microbial cells taken from the plate by pipette tip, lyze the cells 15 min at 96 °C and thereafter cool the tubes on ice. Centrifuge cell walls and other insoluble residue to the bottom of the tube (1 min, 13 000 rpm) and **only take supernatant for PCR**;
2) calculate and prepare PCR reaction mix with final volume of 25 µl using the sequence provided in table. **NB!** Make sure that all the solution in the tube is melted before pipetting! When pipetting small amounts of material follow the pipetting guidelines! Keep the tubes on the ice before inserting them into PCR machine! Right before inserting them into PCR machine mix them well (on vortex mixer) and centrifuge any droplets on tube walls to the bottom of the tube!
3) After the PCR machine has finished the PCR program analyze the amplified DNA on 0.8% agarose gel containing 0.5 µg/ml ethidium bromide (EtBr). To make agarose gel use 0.8 g agarose for 100 ml 1x TAE buffer, mix and heat on hotplate or microwave stirring occasionally until agarose is completely dissolved. Cool the mixture to about 60 °C, add 5 µl EtBr (10 mg/ml) and pour the gel mixture onto the gel plate adding the „comb" (mold piece forming the sample wells). After the gel is set, gently remove the „comb", submerge the gel with its plate into the 1x TAE buffer inside phoresis bath. Transfer 7 µl of sample mixed with **3** µl of loading buffer (6x Mass Loading Dye, Thermo Scientific) into a sample well. Observe that the DNA size marker would also be loaded onto the gel (**3** µl) (GeneRuler^{TM}, 1 kb DNA Ladder, Thermo Scientific). Before turning the electrophoresis machine on examine the placement of the electrodes. After the samples have moved to half the length of the gel turn the machine off and observe and photograph the gel under UV. In the case of positive sample make sure to note if the amplified DNA fragment is of the correct size (623 bp).

Used primers:

gusAF 5´-CCAAAAGCCAGACAGAGT-3´ (1066-1083, positions in gene, T_s=49 °C)
gusAR 5´-GCACAGCACATCAAAGAG-3´ (1689-1671, T_s=49 °C)

PCR program:

1. 94 °C	2 min	
2. 94 °C	30 s	
3. 49 °C	30 s	32 cycles
4. 72 °C	1 min	
5. 72 °C	7 min	

Component	Final concentration in reaction mixture	Amount of component in 25 µl reaction mixture
1. Sterile water	-	... µl
2. 10x PCR reaction buffer [750 mM Tris-HCl, pH8.8; 200 mM $(NH_4)_2SO_4$; 0.1% Tween 20]	1x	... µl
3. 25 mM $MgCl_2$	2.5 mM	... µl
4. 2.5 mM dNTP mix (deoxy ribonucleotides)	0.2 mM	... µl
5. Mixture of praimer 1 ja praimer 2 (each 5 pmol/µl)	0.4 pmol/µl (both praimers)	... µl
7. *Taq* DNA polymerase (0.25 U/µl)	0.02 U/µl	... µl
8. Target DNA (lysate)		2 µl

The test tube selected for analysis (sample, dilution, inoculum size):
Detecting *E. coli* on SLS+MUG plate: Did the inoculation streak light up under UV: yes/no
PCR to test *gusA* gene: positive / negative
If the results acquired by two different methods differ then explain how this might have happened!

Gel electrophoresis analysis of amplified *gusA* gene product:

VII STUDYING THE GROWTH OF MICROBIAL POPULATION

Inside a microbial cell thousands of reactions take place, the results of which are generated energy and synthesized cell components. The summary result of all these reactions taking place inside a cell is the increase in the amount of cell components, which leads to the cell division where two cells are generated from one. The growth of the microbial culture is mainly evaluated by the increase in the microbial cell number or by the increase of the biomass inside a volume. Most microbiological experiments are conducted at the level of microbial population, where inside the culture are many cells.

The most common means of bacterial reproduction is binary fission but in addition to that there are other means of reproduction such as the division of filaments in the case of filamentous growth of actinomyces, the formation of conidia at the end of hypha and also budding which for example is characteristic to *Caulobacter*. As the binary fission is inherent to many strains studied in laboratory, it is the best studied method of reproduction and therefore our following explanations of population growth will be based on that.

Bacteria are grown either inside a closed system as a periodical or ***batch* culture**, into which nothing is added or from which nothing is removed (excepting the gaseous phase exchange) during incubation or inside a **continuous culture** into which in constant speed fresh medium is added and used medium with cells is removed. It is more complicated to grow a continuous culture as it is time consuming to find out the proper dilution speed - the adding and removal of medium and the cell growth have to be in balance and the sterility has to be guaranteed both in adding and removing the medium. During such constant cultivation the microbes keep growing exponentially and the growth rate is equal to the dilution speed.

The cultivations inside closed systems can be done from small sized test tubes to the fermenters sized several hundred liters, while all of them are inoculated with a small amount of inoculum. The growth rate inside the periodic culture is not constant due to the changes happening inside the environment. Namely the concentration of nutrients (and in the case of aerobic bacteria the concentration of oxygen) falls during the growth while the concentration of residual products rises, both of which will cause the fall in growth rate. For these reasons the microbial growth is characterized by four stages: lag phase, log phase (also called exponential phase), stationary phase and death phase. The growth of microbial population inside a *batch*-culture is characterized by growth curve, a graph that will show the changes in the cell number or biomass in time. Knowing the methods for determining the cell numbers or biomass it is possible to analyze the kinetics of microbial growth.

In lag phase takes place the adaption of microbial cell to the new environment, the preparation of cell's metabolic apparatus to the growth, induction of enzymes, synthesis of necessary growth factors and ribosomes. Due to such changes the biomass of the cell grows without any cell division yet. The cell is physiologically active in this phase and the DNA replicates at the end of this phase just before the start of divisions. The length of lag phase is different and depends on the amount of cells in inoculum and their growth phase at the time. It also depends on the differences in chemical composition of old and fresh medium.

In logarithmic (log) phase there exists a direct correlation between the logarithm of the cell number and the time. This is a phase of growth where all the cells in the culture divide actively and in constant intervals meaning the growth rate is constant (balanced growth). In exponential growth phase the bacterial cells are metabolically similar to each other. This is why cells from the middle of this phase are used in different biochemical and

genetic experiments. The time during which the amount of cells double (or put in the other way – the time it takes for a cell to fission) is called **generation time (g):**

$$\boldsymbol{g = \frac{t}{n}},$$

where n – number of generations and t – time.

The generation time is generally constant for each microbial strain and reflects on their genetic potential but is also heavily dependent on environmental conditions. The shortest generation time belongs to *E. coli* grown at optimal conditions. Usual generation times for bacteria are 1-3 hours, but there are also bacteria having 24 hours or more as generation time. The increase in the cell numbers depend on the cells already present so this is a geometric growth. Example:

Number of generations	Exponential presentation	Number of cells (N)	logN
0	2^0	1	0
1	2^1	2	0.30
2	2^2	4	0.60
3	2^3	8	0.90
30	2^{30}	1 073 741 824	9.03

To find generation time by the indirect method a graphic method is used. Where on the x-axis the incubation times and on the y-axis the logarithmic cell numbers or biomass is noted. A growth curve is plotted in this half-logarithmic co-ordinate system on which the exponential growth phase is identified. Through to the points of exponential phase a straight line is fitted. To find a generation time, two points are taken from this line so that the biomass or cell numbers differ two-fold. Out from these points parallel times to x-axis are drawn. The interval on x-axis between these lines shows the **generation time for this specific bacteria grown in these specific conditions.** Let's assume that a culture grew in logarithmic growth phase from 0.2 OD units to 0.4 OD units and the perpendicular lines taken from growth curve show time points 60 min and 90 min on the x (time)-axis, then: g = 90-60 = 30 min.

To find generation time by the direct method an equation based on logarithm of cell numbers is used. The equation is generated on following principles. As explained previously the population growth starting from one cell is a geometrical progression:

$$\mathbf{2^0 \rightarrow 2^1 \rightarrow 2^2 \rightarrow 2^3 \rightarrow 2^4 \rightarrow \ldots \rightarrow 2^n,}$$

where **n** is **generation time**.

With each cell division a new generation is formed in which there are 2 times more cells. So inside an active growth phase the microbial population increases exponentially:

$$\boldsymbol{a)} \quad \boldsymbol{N_t = N_0 \times 2^n},$$

where N_t – bacterial number at some time moment t; N_0 – bacterial number at time moment 0, at the start of the growth; n – number of generations. Taking a logarithm from this we get:

$$\boldsymbol{\log N_t = \log N_0 + n \times \log 2}$$

and then we express the number of cell divisions or number of generations **n**:

$$\mathbf{b)} \quad \boldsymbol{n = \frac{\log N_t - \log N_0}{\log 2}}.$$

Equation to calculate **generation time** (**g**) can be derived if we use correlation provided earlier (g=t/n) and do appropriate substitutions to the previous equation:

$$\textbf{c)}\quad g = \frac{t \times log2}{logN_t - logN_0}.$$

In the case we have the calibration graph showing the correlation between OD and cell numbers (chapter V, Work 7) for a specific bacterial strain we can use the equation c to calculate the generation time in logarithmic growth phase. For example, if in 10 hours the bacterial population increased from 10^3 cells to 10^9 cells, then:

$$g = \frac{10 \times log2}{log10^9 - log10^3} = \frac{10 \times 0.301}{9-3} = \mathbf{0.5}\ \text{h}$$

meaning the population doubles in 30 min

For microbiological analyses it is often necessary to quantify **growth rate (v)**, which is the number of generations in a unit of time:

$$v = \frac{n}{t}$$

or the rate of cell division is the inverse of generation time:

$$v = \frac{1}{g}.$$

Making appropriate substitutions in equation **c),** we get the following equation to determine the growth rate:

$$\textbf{d)}\quad v = \frac{logN_t - logN_0}{log2 \times t}.$$

So the rate of growth is calculated from the inverse of generation time meaning the faster the cells grow the shorter the generation time. According to the previously set example: v = 1/0.5 = 2 h^{-1} (population doubles in an hour).

The growth rate is determined by the genetic features of the cell and the chemical composition and physical parameters of the environment. The length of the log phase depend on the concentration of the growth substrate and the amount of accumulating toxic waste products. At the end of the log phase the number of dividing cells drop and the culture moves to the next growth phase.

Stationary phase is such a state in culture where the rate of bacterial cell growth is equal to the rate of bacterial cell death and the density of the cell population does not change in time. Cells survive mainly on the stored nutrients and have little metabolic activity. For example the cells that are capable of forming endospores go to sporulation in this phase. The culture often reaches this phase due to the end of the growth substrate from the environment, for aerobic organisms it can happen in the limited O_2 environment and it can happen due to accumulation of toxic waste products or strong changes in environmental pH. The length of stationary phase can be very long and it is characteristic to the microbes in nutrient sparse environments.

In death phase, where the logarithmic decline of live cell number takes place, the cells lyze due to their own enzymes. There are microbes who do not enter this phase being able to persist for a long time at hunger conditions.

QUESTIONS

1. What is the definition of bacterial growth?
2. Which methods of bacterial reproduction are known?
3. What is a periodic culture?
4. Characterize the growth phases in a periodic culture?
5. What is the length of lag-phase dependent on?
6. What causes the end of log phase?
7. What is the definition of balanced growth?
8. Define generation time. What does it depend on?
9. How do you find the generation time for a microbial population?
10. How are the generation time and the growth rate correlated? Which are their units?
11. Why is a half-logarithmic co-ordinate system used to evaluate the microbial population growth?
12. Can one find generation time in each growth phase?
13. Find the generation time and growth rate when in 12 h microbial culture grows from 5 x 10^2 cells/ml to 1 x 10^8 cells/ml?
14. If there are 4 doublings of biomass in an hour then what is the generation time?
15. How large is the progeny of one cell after 8 h when the generation time is 20 min?
16. At 2.00 o'clock 10^3 cells were inoculated into the culture. Lag phase lasted for 30 min. At 7.30 the culture with density 10^6 went to stationary phase. How many generations happened? Find the generation time!
17. In a potato salad is a single pathogenic cell with generation time of 20 min (for this environment). What is the cell number in 4 hours?
18. When is the generation time equal to the doubling time of the biomass?
19. How long are the generation times for microbes in nature? Explain!
20. Characterize stationary growth phase.
21. Characterize death phase.
22. How would you determine if the cells in culture are dead?
23. If we express CFU/ml and the absorption of light in OD at the same graph, then do the 1) maximums of both arrive at the same time? Explain!
    2) is the start of death phase simultaneous?
24. How does the continuous flow culture differ from periodic culture?
25. What determines the growth rate in continuous culture?
26. In which growth phase is the continuous culture?

PRACTICAL WORKS AND RESULTS

Work no. 1. Cultivating *E. coli* wt in 0.2% casamino acids (CAA) or glucose (Glc) containing minimal medium and in LB medium and constructing a growth curve (work for the whole group)

Material to be studied: *E. coli* wt culture in log phase in 0.2% CAA/glucose minimal medium or LB medium.

1) Prepare minimal medium into 1 l Erlenmeyer flask (final volume in flask 200 ml) in such a way that the concentrations of M9, microelements and carbon and energy source concentrations would be 1x, 1x and 0.2% respectively. Use concentrated sterile starting solutions - 20% CAA or Glc, 10x M9 and 400x microelement mixture;
2) pour into another sterile 1 l flask 200 ml sterile LB medium;
3) measure the OD_{580} of inoculum and inoculate with *E. coli* such as the starting **OD ~ 0.1**;
4) cultivate on thermostat-shaker at 37 °C;
5) under the laminar flow hood after every 15 min take samples using automated pipette and sterile pipette tips from the cultivation flask to measure OD;
6) enter the data into *Excel* spreadsheet;
7) using *Excel* plot the growth curve both in arithmetic and half-logarithmic co-ordinate system noting OD values on y- and incubation times on the x-axis;
8) find out **indirectly** the generation time for *E. coli* wt by the doubling of the biomass;
9) using the calibration graph constructed in chapter V that shows the correlation between cell number and OD also find the generation time.

VIII PURE CULTURES OF MICROBES AND THEIR IDENTIFICATION

A pure culture or axenic culture is a culture where cells of only a single microbial species are present. Isolation of a pure culture, controlling its purity and avoiding contamination are crucial microbiologists work. Pure cultures could be isolated only after introduction of agar plates by Koch.

In order to obtain separately growing colonies that are sufficiently far apart and can be individually studied and subcultured, the inoculum is **streaked** on an agar plate with an inoculation loop from solid medium or with a pipette or spatula from a liquid medium (see chapter III). In the first case, the plate is streaked with an inoculation loop on the surface of ¼ of the plate, then the loop is sterilized and another quarter of the plate is inoculated with it by carrying over some cells from the first quarter. Then the loop is once again sterilized and the third quarter is inoculated with some cells from the last streak etc. There are several ways to do a streaking, but the method described is the best to ensure that single colonies are formed.

If the streaking is carried out with a spatula, the microbes are pipetted on the first agar plate from the liquid culture and spread. The same spatula is then used for inoculating few other sterile agar plates, whereas in the meantime it is not sterilized. This way individual colonies will grow on the latter plates. A cheaper possibility is to dilute the initial sample and then plate an appropriate dilution with the spatula.

The purity of the isolate is checked carefully both visually and microscopically.

Unfortunately, not all colonies grow from a single cell. Some microbial cells are sticky and if cells of different species are like that, we may not get pure colonies from the first streaking. If low enough number of foreign cells are present, a mixed colony may not be visually detected. Further purification should be carried out on another medium. It should be complete medium to ensure the maximal growth of all cells. Also, the incubation time should be long enough, so the slower growing contaminating cells can form colonies.

To identify and classify an unknown microbe, as many as possible phenotypic (morphological, biochemical, physiological, chemical, serological, ecological etc.) and genotypic characteristics (DNA G+C%, the nucleotide sequence of the whole genomic DNA or some specific regions/genes) must be determined and then compared with known reference strains from the databases. This approach is called **polyphasic classification**.

The tests for identification must be standardized, fast and as cheap as possible. The number of basic tests necessary to identify different microbial groups can be very different and thus it is necessary to make preliminary tests to classify microbes into one or the other group. It is started from a broader category and moved to more specific ones. Based on the results of the preliminary tests, tests that are unnecessary for a certain microbial group can be skipped.

A. GENOTYPIC CHARACTERISTICS. 16S rRNA GENE SEQUENCE

Genotypic characteristics are more stable (for example, not affected by growth conditions or cell age) and display the evolutionary heritage of the organisms (phylogenesis). The phylogenetic approach is the basis of contemporary prokaryotic systematics. Despite the fact that determination of the whole genome nucleotide sequence or sequencing is increasingly more available and affordable, it is not rational to use it for routine identification. The most well-recognized and common approach is amplification of the housekeeping genes with PCR, sequencing and comparison of these sequences with the ones in the database. These so-called „**molecular chronometers"** can be genes that fulfill the same function in all organisms, have conserved sequences, are large enough to contain adequate amount of information and don't go through horizontal transfer. The **16 rRNA** gene is most commonly used, but also genes that code 23S rRNA, ATPases, DNA gyrases, RNA-polymerases, tRNAs, ribosomal proteins, cytochromes etc. Also, 16S rDNA-23S rDNA intergenic region, which is not highly conserved, is used to distinguish lower taxons.

The required genome region of the microbial strain to be identified is amplified with specific primers and the obtained DNA fragment is then sequenced. The 16S rRNA gene has more and less conserved regions. The more conserved the region, the more universal primers can be designed for that region and the more different microbes it can be used for. If the 16S rRNA gene sequence of the studied microbial strain is more than 97% identical with a sequence in the database, it is highly likely that these organisms are in the same genus. At the same time, there are species that have up to 5% intraspecies 16S rRNA genetic variability and species that have more than 99% homologous genes. Due to that, nowadays often analysis of two or more gene sequences (multilocus sequence analysis, MLSA) with bioinformatic tools is used for determining phylogenetic relationships of bacteria. Several other methods have been developed due to rapid progress of metagenomics that examine different combinations of alleles (of mostly housekeeping genes) in a genome (multilocus sequence typing, MLST) or the distribution of certain length oligomers in a genome or that search for genome-specific oligomers etc., and according to these group bacteria into taxonomic units. Analogous method with DNA-DNA hybridization is using genome-to-genome distance calculator (GGDC) which needs special computer programs and high computational power.

Bacterial strains are considered to be one species according to other genotypic characteristics if their genome G+C content is less than 5% different, if their genomic DNAs hybridize for at least 70%, and if their amino acid and average nucleotide identity is above 95%.

A prokaryotic nomenclature with an example of *Pseudomonas putida* is here presented:
Kingdom - *Bacteria*
Phylum - *Proteobacteria*
Class - *Gammaproteobacteria*
Order - *Pseudomonadales*
Family - *Pseudomonadaceae*
Genus - *Pseudomonas*
Species – *putida*

Current bacterial nomenclature can be found in http://www.bacterio.net/ and the minimal requisites to define a new species in http://www.bacterio.net/-minimalstandards.html.

B. PHENOTYPIC CHARACTERISTICS

1. MACROMORPHOLOGICAL CHARACTERISTICS

Single uniform colonies are inspected visually and it is important to note the medium and incubation conditions. The colonies have several traits that can be used for characterization.
The colonies can have circular, rhizoid, irregular, filamentous etc. **shape.**

The size of the colonies is usually between 5 to 10 mm and it is markedly dependent on the closeness and age of the colonies. Some microbes have punctiform colonies with a diameter less than 1 mm.

The surface of the colonies can be smooth, rough, rugose, veined etc. It can even differ in one microbial strain: smooth (S) or rough (R). For some microbes, it is linked with pathogenicity.

The elevation or **cross-section** of the colony can be flat, raised, convex, umbonate, crateriform etc.

The color of the colonies can be off-white (these are usually described as colorless), white, yellow, purple etc. Pigmentation is also dependent on the growth environment. It the medium around the colonies does not stain, the pigments are insoluble in water. Some microbes produce water-soluble pigments too. Typical examples are some representatives of the genus *Pseudomonas*, who form blue (pyocyanine, *P. aeruginosa*), green (chlororaphine, *P. ahlororaphis*), orange (phenazine-1-carboxylic acid, *P. aureofaciens*) and green-yellow UV-fluorescent pigments (fluoresceine or pyoverdine, *P. fluorescens*). Pseudomonads producing fluorescent pigments or siderophores are identified on iron-deficient KingB medium, as the production of these pigments is activated on that medium.

The consistency (and texture) of the colonies means if they are dry, mucous (also depends a lot on the chemical composition of the medium), viscid, friable. In the latter case, it is important to examine the presence of substrate mycelium (the colony does not detach from the medium). All these morphological characteristics of the colonies change during the growth and depending on the nature of the medium.

Microbes are also characterized by the features they present when growing in **liquid medium**. The growth is marked by the formation of turbidity, pellicle, mucous filaments or flocculent sedimentation, whereas all these features can be characteristic to a specific microbial strain. Microbial growth in liquid environment can cause excretion of pigments into the medium (for example colorful metabolites), formation of gas and odor. Profuse formation of gas is identified with Durham tubes (see chapter VI).

2. CELLULAR FEATURES

The cellular features of microorganisms are mostly studied with microscopy. The most important features are Gram-stain reaction, cell shape, size, motility, presence and positioning of flagella, cell aggregation, presence and positioning of spores and capability to store nutrients. Cell mobility, sporogenity and stored lipophilic compounds can be studied with cultural methods.

The motility can be studied with a „hanging drop" method (see chapter I) or the microbes can be tested in semisolid agar deep, where agar concentration is 0.2-0.3% to prevent the „swimming" of microbes. If the microbe is motile, the medium column around the inoculation line becomes turbid. Weak agar media based on ordinary nutrient broth, Hugh-Leifson media with glucose (there are not many non-utilizers of glucose) and also the basal media for carbohydrate utilization can be used. The motility can be evaluated also on the media plates. Cells are taken from 1.5% agar plate and inoculated with a toothpick to 0.3-

0.5% agar plate. The growth zone around the inoculation is monitored after overnight incubation.

The presence of **endospores** can be determined by killing vegetative cells in 15-20 min at 80 °C followed by incubation in thermostat for spore germination. R2A medium is well-suited solid medium for studying endospores as they can be detected in the cells grown on that medium already after overnight incubation. For that purpose, an endospore smear is made from the cells for microscopy (see chapter I).

The formation of **lipophilic storage substances (PHB)** can also be studied both microscopically (see chapter I) and culturally. The cells are cultivated on a nutrient agar containing glucose for 3-5 days. The accumulation of storage substances is identified by staining the colonies on the medium plate with sudan black B ethanol solution for 20-30 min, followed by decolorization for 1 min with 96% ethanol. Colonies stained dark blue or black prove the accumulation of PHB.

3. BIOCHEMICAL TESTS

In previous chapters, we studied biochemical activity of bacteria, expressed in growth and replication. The whole biochemical process of transformation occurring both intra- and extracellularly takes place in the presence of biological catalysts – enzymes. As biochemical tests are an important criteria in taxonomy, some of them are discussed in the present topic.

3.1. Carbon sources.

The utilization of various C-sources (organic acids, sugars, alcohols etc.) by different microbial groups is an important identifying biochemical characteristic. In order to utilize a certain C-source, the microbe must have enzymes necessary for each metabolic step (including transport to the cell) of that substance, which is ensured by the presence of genes encoding these enzymes.

S. A. Koser made the first attempt to identify microbes based on their ability to grow on different C-sources in 1923. He discovered that enterobacteria groups *aerogenes* and *coli* have differences in **citrate** utilization. The test was later refined by J.S. Simmons who added indicator dye and agar to the medium (see chapter IV) and this is known as Simmons citrate agar. Citrate test is used for the identification of enterobacteria as a part of **IMViC test** series, where **I** stands for indole test, **M –** methyl red test, **V –** Voges-Proskauer test and **C –** citrate test.

An important characteristic in identifying microbes is their capability to obtain energy from **the sugars** either by fermentation or respiration. **Fermentation** is an anaerobic process to degrade organic substances, where the organic substance is terminal electron acceptor and acids, alcohols, butanediol and gases are usually formed as end-products. The amount and nature of the fermentation products depend on the microbe and cultivation conditions. Following fermentation types are distinguished: lactic acid (*Lactobacillus, Streptococcus*), alcohol (*Saccharomyces, Zymomonas*), propionic acid (*Propionibacterium*), 2,3-butanediol (*Enterobacter, Bacillus*), mixed acid (*Escherichia*) or butyric acid (*Clostridium*) fermentation. As seen, there are different fermentation types among enterobacteria (see also chapter VI). This is the reason for two reactions in the aforementioned classic **IMViC test**, one of which is **methyl red test (M)**, where the microbe turning glucose into acids causes indicator color change to red (pH 4.0). The other one is **Voges-Proskauer test (V)** which identifies accumulation of 2,3-butanediol into the medium. The determination is based on the identification of its precursor acetoin (acetyl methyl carbinol) with Barritt's reagent (contains α-naphthol in ethanol and 40% KOH) and after exposing in atmospheric oxygen forms pink complex of creatine:

:

$$glucose \rightarrow 2\,pyruvate + CO_2 \rightarrow \alpha - acetolactate + CO_2 \rightarrow acetoin \rightarrow 2,3 - butanediol$$

$$acetoin + \alpha - naphthol \xrightarrow{40\%\ KOH, ethanol} diacetyl + creatine.$$

To determine the type of metabolism, usually **oxidation/fermentation (O/F) test** is performed, where glucose – the most commonly utilized sugar among chemoheterotrophs - is the main C-source (see APPENDIX II; **Hugh-Leifson medium**). The concentration of peptone has to be minimal in that medium to prevent formation of basic products that can obscure acidic fermentation or oxidation products of glucose. It is recommended to substitute 0.2% peptone with 0.04% $(NH_4)_2HPO_4$ to prevent alkalification of the environment. Indicator dye bromothymol blue (see chapter II) is added to the medium to observe pH change, but it can inhibit the growth of GP microbes. The O/F test for identification of fermentation is carried out under agar cap to avoid oxygen diffusion to the medium. A parallel test tube without the agar cap shows aerobic catabolism of glucose. If extensive amount of gaseous fermentation products (CO_2 and H_2) accumulate, the agar cap will be pushed up in the test tube. Optimal time for monitoring the fermentation test is 48 h, as alkaline degradation products of the substrate formed in longer exposure can obscure the acidic reaction. The color change of the indicator dye will be apparent in both capped and uncapped test tube if fermenters are present. Aerobic utilization of glucose and other sugars can be decided by the accumulation of acid in the medium. Usually, the utilization of various sugars by microbes is tested with **basal media for carbohydrate utilization** (see APPENDIX II), which has 2 different indicator dyes in the medium for more sensitive detection of pH changes. The production of acid from sugars needs longer monitoring for at least 1-3 weeks as many microbes metabolize first amino acids and only then sugars. As many gram-negative microbes (*Pseudomonas, Rhizobium, Azotobacter, Agrobacterium*) degrade hexoses rather by the Entner-Doudoroff pathway than glucolytically, the accumulating acidic degradation products (6-phosphogluconate, 2-keto-3-deoxy-6-phosphogluconate) make the utilization of sugar especially well tractable.

3.2. Enzymes.

Enzymes are important indicators of cellular activity. They take part in the synthesis of cellular matter and degradation of unnecessary products. As different enzymes are related to specific substrates, the cell has to have a wide range of enzymes. Existence or deficiency of many enzymes is species-specific, which makes enzymatic tests important for identification.

3.2.1. Extracellular hydrolytic enzymes. Before one or the other organic molecule can be used as a C- or energy source, it must be transported into the cell. Some microbes produce and secrete hydrolytic enzymes to the environment to assimilate polymeric organic molecules, so they are degraded outside the cell. The hydrolysis products are transported to the cell as mono- and dimers. Extracellular enzymes are more typical to GP microbes. Examples of extracellular enzymes are polysaccharide-degrading **glucosidases**, protein-degrading **proteases** and lipid-degrading **esterases.** Also, penicillin-degrading penicillinase is extracellular in GP bacteria, but periplasmic in GN bacteria.

The most abundant polysaccharide in the nature – **cellulose** is composed of long linear chains of glucose units linked by β–1,4–glucosidic bond, which is degraded by **cellulases** to the lowest disaccharide intermediate – cellobiose. Cellulolytic activity is identified by culturing the cells on carboxymethylcellulose and it is characteristic to many fungi, plant pathogens, clostridia and bacilli.

β-1,4-glucosidic activity which carries out degradation of cellobiose in microbes is measured on esculin-based medium. Esculin is UV-fluorescent glucoside, in which glucose is linked to aglucone esculetine with β-1,4-glucosidic bond. In case of esculinase activity,

esculetine or dihydroxycoumarin is released which reacts with iron in the medium, causing black sedimentation and loss of fluorescence.

The hydrolytic degradation of two forms of **starch** – water-soluble amylose (β-1,4-glucoside) and insoluble amylopectin (branching with α-1,6-glucosidic bonds in addition to β-1,4-glucosidic bonds) – by **amylases** produces smaller oligosaccharides, dextrines, maltose or glucose residues. To test amylase activity, the microbe is cultured on the starch medium and later the surface is treated with Lugol solution. The characteristic blue color is caused by iodine binding to the helical structure of amylose. Amylase activity is characteristic to many bacilli, pseudomonads, actinomycetes and microfungi.

Protein molecules are degraded by **proteolytic enzymes** to oligopeptides, which are later intracellularly degraded to amino acids. Exoproteases are typical for the members of *Bacillus* family and many microfungi. One method for identification of proteases is observing degradation of milk protein **casein.** Milk is a perfect medium for microorganisms as it contains up to 4% fat, 5% lactose, 3% protein, 1% vitamins, minerals and 87% of water. Casein makes up 80% of the milk proteins. Casein is thermally resistant and not coagulated by heat, but coagulates in acidic environment. Casein is a colloidal suspension of insoluble calcium salt (Ca-caseinate) and if hydrolysis takes place, casein particles are lost and transparent zones of hydrolysis form around inoculation line. The size of these zones illustrates the magnitude of caseinolytic activity.

Proteolytic activity is also identified with **gelatin**, which is a soluble protein obtained from various animal tissues (connective tissues, tendons, cartilage, bones, skin). Hydrolysis of gelatin takes more time (in case of low proteolytic activity it can take even up to a month) and it is determined with a medium where gelatin acts as a gelling agent.

Some microbes also produce DNA-degrading **deoxyribonuclease** (DNAse). This enzyme is identified with DNA-containing agar medium, where DNA degradation is determined by treating the plate with either weak acid or 0.1% toluidine blue. Intact DNA is insoluble in weak acid and precipitates, whereas DNA degradation products are soluble and the culture with DNAse becomes transparent around the inoculation line. Toluidine blue stains intact DNA blue and a pink zone is formed around the inoculation line of a culture producing DNAse.

3.2.2. β-galactosidase. One important test enzyme is β-galactosidase which catalyzes lactose catabolism by breaking β-1,4-glucosidic bond, whereas β-galactose and β-glucose are formed. Although lactose utilization is visible with various selective-differential media (Endo, MacConkey) and artificial substrates (see chapter VI), the activity of this enzyme can also be determined with a fast test. For that, an artificial colorless substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG), is used:

$$\boldsymbol{ONPG + H_2O \xrightarrow{\beta-galactosidase} galactose + o-nitrophenol\ (yellow)}$$

The presence of enzyme is proved by the formation of intense yellow color of nitrophenol. To utilize lactose as a sole C- and energy source, the cells need not only β-galactosidase (encoded by *lacZ* gene) but also permease (encoded by *lacY* gene) which transports lactose into the cell.

3.2.3. Tryptophanase. Some microbes have an ability to degrade amino acid tryptophan to indole, pyruvate and ammonium with an enzyme tryptophanase:

$$\boldsymbol{tryptophan \xrightarrow{tryptohanase} indole + pyruvate + NH_3}$$

$$\boldsymbol{indole + p-dimethylbenzaldehyde \xrightarrow{HCl,amylalcohol} rosindole\ dye\ (cherry\ red)}$$

The activity of tryptophanase is identified with a non-metabolized and accumulating **indole** which forms a red complex with p-dimethylaminobensaldehyde in Kovacs reagent. To carry out the test, the cells are cultured on a medium containing tryptophan (LB or tryptic soya broth, TSB). This test is also known as **indole test** and it is a part of IMViC test series that is used for distinguishing enterobacteria (see above). The importance of this test lies mostly in distinguishing GN fermenters.

3.2.4. Urease. A proteinous waste product urea (carbamide) of vertebrates is hydrolyzed by an enzyme urease that is produced by some microbes, and thus this reaction is used for their identification. As N-C bond is degraded in carbamide, CO_2 and ammonia are released. As the latter accumulates and reacts with water, NH_4OH is formed which makes the medium alkaline (see also chapter IV). It is an important test for distinguishing lactose non-fermenting enterobacteria.

3.2.5. Arginine dihydrolase. Arginine is degraded in bacteria via two known pathways: arginine-urease and arginine-deiminase pathways. The latter is called arginine dihydrolase (ADH) which is actually comprised of three enzymes: arginine deiminase, ornithine carbomyl-transferase and carbamide kinase:

$$\boldsymbol{arginine + H_2O \xrightarrow{arginine\ deiminase} citrulline + NH_3}$$

$$\boldsymbol{citrulline + P_i \xrightarrow{ornithine\ carbamoyl-transferase} ornithine + carbamoylphosphate}$$

$$\boldsymbol{carbamoylphosphate + ADP \xrightarrow{carbamate\ kinase} ATP + CO_2 + NH_3}$$

The activity of ADH is identified with a pH increase in the medium as NH_3 is released. This is monitored by the color change of phenol red in anaerobic environment. ADH detection is important for distinguishing *Pseudomonas syringae* group from other non-fermenting GN bacilli, and also to distinguish different species/strains in that genus.

3.2.6. Cytochrome c oxidase (oxidase test) catalyzes electron transfer from reduced cytochrome c (cyt c) to molecular oxygen, and as a result of that oxidized cyt c and water are formed. The determination of this enzyme is based on the utilization of artificial substrate dimethyl-*p*-phenylenediamine which is oxidized by the oxidase and in the presence of oxygen to a violet indophenol blue:

$$\boldsymbol{2\ red.cyt\ c + 2H^+ + \frac{1}{2}O_2 \xrightarrow{oxidase} 2\ ox.cyt\ c + H_2O}$$

$$\boldsymbol{2\ ox.cyt\ c + dimethyl-p-phenylenediamine \rightarrow Wurster\ blue + 2\ red.cyt\ c}$$

The test is important for distinguishing GN bacilli and cocci.

3.2.7. Catalase. Microbes growing in aerobic conditions have to be capable of degrading toxic oxygen species (see chapter IV). Catalase degrades hydrogen peroxide to water and oxygen. Bacteria, who are not capable of using oxygen, are not producing catalase. To identify the activity of catalase, the microbial cells are dispersed in H_2O_2 and formation of oxygen bubbles are registered. It is recommended to use young cells, not older than 24 hours for the test.

The catalase test is important for distinguishing morphologically similar but metabolically different species (gram-positive cocci and bacilli).

3.3. Denitrification.

If oxidase and catalase described above characterize bacteria with oxidative metabolism, then denitrification proves anaerobic respiration. Terminal electron acceptor in denitrification process is nitrate, which is reduced to gaseous products N_2O and N_2:

$$\mathbf{NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2}$$

For the identification of some bacterial groups (*Pseudomonas, Bacillus, Alcaligenes*) it is important to verify their denitrification capacity. The generation and release of gaseous nitrogen can successfully be monitored for that purpose (e.g. Hiltay medium, APPENDIX II; see also chapter V 1.3). N_2 is released as bubbles if microbes are cultured in a liquid medium containing nitrate, where oxygen permeation is hindered. Released N_2O does not form bubbles, because it is water soluble (see also chapter V).

3.4. Salt tolerance.

Bacteria are in general capable of living in a hypotonic or hypo-osmotic (extracellular salt concentration is lower than intracellular) environment due to their rigid cell wall. Hypertonic or hyperosmotic environment (extracellular salt concentration is higher than intracellular) on the other hand causes plasmolysis and cell death. Some bacteria (halophiles) are able to tolerate high salt concentrations in their growth environment (15-30%). Microbes surviving at moderate salt concentrations (up to 11%) are called halotolerant. Studying salt tolerance is necessary for distinguishing gram-positive cocci and bacilli and also for the distinction of some gram-negative bacteria in one genus. Salt-free nutrient agar media with added NaCl resulting in different final concentrations of the salt are used for the test.

4. NOVEL METHODS AND TESTING SYSTEMS FOR IDENTIFICATION OF BACTERIA

Traditional identification methods are time-consuming and laborious, thus commercial testing systems for identifying microorganisms have been develop to facilitate the work of microbiologists. Some of the more well-known and used multitesting systems are, for example API, MINITEK, BBL CRYSTAL, BIOLOG etc. These quick methods are especially popular in medicine, as fast identification of pathogens can be a matter of life and death. Due to that, the databases of multitesting systems are largely with clinical nature. A selection among trivial identification tests have been made in commercial tests that comprise a so-called minitesting system – a set of compartmentalized tests. The number of comparable reactions in different sets is different, ranging from 12 reactions in Enterotube to 94 on BIOLOG plates. There are systems identifying specific microbial groups, for example special API (*Analytical Profile Index*) testing systems for enterobacteria (API 20E), non-fermenting GN bacteria (API 20NE), coryneform bacteria (API CORYNE), strains of *Bacillus* genus (API 50 CHB) etc., but there are also more universal testing systems enabling to determine very different microbes, like BIOLOG. In some cases, the results can be obtained as fast as 4 hours after incubation, but in general after 24 to 48 hours. The results are analyzed with special computer programs.

So far, the most substantial database of reference strains (more than 2500 aerobic or anaerobic strains of bacteria, yeasts and fungi) is BIOLOG GenIII system (*Phenotype MicroArrays for Microbial Cells*, http://www.la-biosystems.com/index.php?id=317), which enables to identify also environmental species in addition to clinical isolates. Wells of microplates contain 94 different anhydrous carbon sources together with redox dye tetrazolium blue. The redox dye is irreversibly reduced to violet formazan in the course of substrate oxidation, which is registered with multiscanning spectrophotometer. A metabolic

pattern is obtained from the utilization of different carbon sources that the computer compares with the metabolic patterns of test strains in Microlog database and thus the unknown strain is identified. Unlike most identification systems that evaluate the reaction by pH change, BIOLOG correlates formation of formazan with the intensity of substrate utilization which enables to also evaluate the utilization of those substrates that are not causing pH changes when degraded or noticeable biomass increase. Successful identification is ensured by pre-culturing a pure culture on a BUG medium required in BIOLOG systems. The inoculum liquid of bacteria must be prepared according to the instructions provided with the testing system. Only young and metabolically active (not over 24 h) cells are used for inoculation. BIOLOG plates are incubated for 24-72 h. At the same time, this system has been used for strains with very slow growth and those that have previously been considered unculturable.

In recent years, MALDI-TOF (matrix-assisted laser desorption-ionization time-of-flight mass spectrometry) method has been used more and more for identification of pure cultures, especially strains from clinical samples. It enables to identify the phylogenetics of an unknown strain in less than an hour. For analysis, a matter of one colony is mixed with a matrix substance (2,5-dihydroxybenzoic acid, sinapic acid, ferulic acid etc.), the sample is ionized with a laser and obtained ions with positive and negative charge are detected with mass-spectrometer. Thus, a peptide mass-spectrum of a strain is obtained, which is compared with the spectra of reference strains in the database. The method has been mostly applied for the analysis of clinical strains where fast identification of a pathogen ensures appropriate treatment and restriction of its spreading. Due to that, the databases of clinical strains are more substantial than those of environmental strains. Still, determination of genetically very close strains can be imprecise (*E. coli* versus *Shigella*). At the same time, if the equipment is available, the net cost of a sample is low, it takes about 1.5 h to analyze 96 samples, the method is sensitive and specific, the method enables to compose own databases, and both bacteria and fungi can be analyzed.

Determination of chemotaxonomic traits is also the basis of Sherlock™ Microbial Identification System products by company MIDI, that distinguishes bacteria by their fatty acid methyl ester (FAME) or phospholipid-derived fatty acid (PLFA) profiles (http://www.midi-inc.com/pages/microbial_id.html). The costs related to purchasing the equipment (gas chromatography) and software are again high, but later a single analysis of a sample is not. Chemical structure of fatty acids present in the cell cytoplasmic membrane and outer membrane (GN) are very stable characteristics and with a database it is rather easy to identify microorganisms. The fatty acids extracted from the cells are derivatized to respective methyl esters before gas chromatography and multidimensional analysis is used for the interpretation of the results.

5. ANTIBIOTIC SUSCEPTIBILITY TESTING OF BACTERIA

Microbial groups are also distinguished based on their antibiotic sensitivity. This topic will be covered in more detail in chapter IX.

C. IDENTIFICATION OF AN UNKNOWN MICROBE

Identification of an unknown microbial culture is one of the most accountable and interesting steps of work for a microbiologist. Morphological simplicity of a prokaryotic cell and insignificance of characteristics separating one microbial group from another in determination of all bacteria make their classification complicated. Previously described genotypic and phenotypic tests help to identify an unknown bacterium, but often these characteristics don’t

even allow to identify the genus. Skillful use of field guides is of great help. Bergey (1860-1937) was an American bacteriologist who was the first to systemize bacteria based on their phenotypic characteristics to orders, families, genera and species. First Bergey's guide was published in 1923. If publication of the first *Bergey's Manual of Systematic Bacteriology* (published in 1984-1989) was considered a milestone of great progress at the time, it is now obsolete. In 2001-2012, 5 volumes of Bergey's *Manual of Systematic Bacteriology* (2nd edition) was published where microbes are systematized based on the similarity of their genetic material, more specifically based on their 16S rRNA gene sequence:
Volume 1 (2001): The *Archaea* and the deeply branching and phototrophic *Bacteria*;
Volume 2 (2005): The *Proteobacteria* (gramnegative bacteria):
2A: Introductory essays
2B: The *Gammaproteobacteria*
2C: Other classes of *Proteobacteria*
Volume 3 (2009): The *Firmicutes* (mostly grampositive bacteria)
Volume 4 (2011): The *Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes*
Volume 5 (2012): The *Actinobacteria* (grampositive bacteria)
Classification based on phylogenetic kinship allows to position bacteria with very different phenotypic features into one group (e.g. genera *Pseudomonas* and *Acinetobacter* are both in the order of *Pseudomonadaceae*) and thus other genetically determined characteristics are needed for identifying a species, and these are also present in the new edition of *Bergey's Manual of Systematic Bacteriology.*

QUESTIONS

1. What is the first step to start identifying microbes?
2. What is pure culture and how can one be obtained?
3. What is polyphasic classification?
4. What kind of genes are suitable as so-called molecular chronometers?
5. What cultural characteristics can be used for identifying microbes?
6. How can motility and presence of endospores be tested in a culture?
7. How is utilization of sugars tested?
8. What is fermentation?
9. Why is it optimal to observe the results of fermentation test two days after inoculation?
10. What is IMViC test and which microbes does it help to distinguish?
11. What is the importance of exoenzymes and name some?
12. Which class of enzymes do exoenzymes belong to?
13. How can the activity of tryptophanase of a microbe be determined?
14. What does the medium have to contain to be suitable for indole test?
15. How can the utilization of lactose by a microbe be determined?
16. Is β-galactosidase test equal to acid formation test on lactose?
17. What does so-called oxidase test show?
18. Which microbial groups (gram reactivity) is the oxidase test important for distinguishing?
19. How indophenol blue is formed in an oxidase test?
20. Which respiration types do you know and how do they differ?
21. What is denitrification?
22. Why does the gas accumulated in the denitrification process not show that it is a fermentation process?
23. What methods are available for rapid identification of microbes?
24. What is the use of BIOLOG testing system for identification based on?

PRACTICAL WORKS AND RESULTS

A. OBTAINING A PURE CULTURE

1) Streak a plate of a microbial strain given by the supervisor on a **nutrient agar** plate using an inoculation loop and incubate at room temperature for seven days.
2) If uniform colonies grow from the colony picked for streaking, it is highly likely that the culture contains cells of a single microbial strain;
3) use an inoculation loop to pick one separately positioning colony and inoculate a **nutrient agar** plate (dense inoculation for obtaining a large biomass). This will be so-called **working plate** for the following studies. Incubate the plate for at least 1-2 days at 23-30 °C.

B. MACROMORPHOLOGICAL AND CELLULAR CHARACTERISTICS

1) Characterize a colony of the studied strain based on the macromorphological characteristics (**size, elevation, margins, consistency, color**). Fill the table with the results obtained here and hereafter (see below);
2) Make a Gram stain of a culture **not older than 24 hours** and characterize the cells of the strain microscopically (shape, size, aggregation). Also, perform a quick KOH test (see chapter I).
3) To identify the capability to form endospores, inoculate some cells with an inoculation loop to a test tube containing **nutrient broth** (the inoculation has to stay clear). Incubate at 80 ºC for 20 min and then at 30 ºC for 24 hours. The endospores were present if the broth becomes turbid indicating growth.
4) **Motility** of the cells can be observed later in semisolid media used for biochemical tests, like Hugh-Leifson medium and the basal media for carbohydrate utilization.
5) Characterize **the growth in liquid medium** (ability to grow at different temperatures, flocculating/uniform biomass).
6) To identify **fluorescent pigments,** make a streak-plate of the isolated strain on **King B medium** plate. As a test strains, culture cells of ***P. fluorescens, P. aeruginosa*** or ***P. putida.*** Monitor the accumulation of fluorescent pigments under UV-light near the inoculation line.

C. BIOCHEMICAL AND PHYSIOLOGICAL TESTS

1. TESTS FOR CARBON SOURCES

1) Make a streak-plate on **Simmons agar** to identify the **utilization of citrate**. Incubate at 30 °C. Observations can be done after 2-7 days. Monitor the growth and color change of the medium (see chapter II).
2) Make stab cultures to 2 test tubes with **Hugh-Leifson medium**. On one culture, pour over a cap of water agar (2 ml). Examine the culture after 2-7 days. Monitor the growth, growth around the inoculation line (motility), color change of the medium and accumulation of gas under the agar cap.
3) Make stab cultures to the **basal media for carbohydrate utilization with different sugars.** Examine the cultures after 7-14 days. Monitor the growth, growth around the inoculation line (motility) and color change of the medium.
4) To determine the fermentation type, inoculate **MP-VP broth**. After 2-7 days of incubation perform a **Voges-Proskauer test.** Use Barritt reagent: take 0.6 ml of

component **A** and 0.2 ml of component **B**. Mix and wait for 20 min. Red color appears if the reaction is positive. **α-naphthol is cancerogenic and very labile! The work must be carried out under the fume hood!** Use *E. aerogenes* cells as a positive control (one positive control for the whole group).

2. TESTS FOR ENZYMES

1) ***β*-galactosidase.** Grow an overnight culture of the sample cells in **LB broth containing 0.2% lactose.** Pipette 1 ml of cell suspension to an eppendorf tube, centrifuge the cells to the bottom (3 min, 6000 rpm), remove the centrifugate with a pipette, add 350 µl of phosphate buffer (pH 7.0) and disperse the cells in it. The cells are permeabilized with 5 µl of chloroform and 10 µl of 0.1% SDS (Na-dodecylsulphate). Mix and incubate at 30 °C for 5-10 min. Add 80 µl of **ONPG** solution (4 mg/ml) and incubate further at the same temperature. Observe the **formation of yellow color with a positive reaction**. Use *E. coli* cells as a positive control for β-galactosidase activity (one positive control for the whole group).

2) **Amylase** activity is identified with making a streak-plate of the sample cells on one half of **starch** agar medium plate. *B. subtilis* cells are used as a positive control. Incubate the cultures for 1-2 days at 30 °C. Pour 1-2 ml of Lugol solution over the plate and examine if colorless zones are formed around the inoculation lines. Explain.

3) **β-glucosidase** activity is identified with streaking an agar slant containing **esculin**. Monitor the growth and color change of the medium. Use *Erwinia carotovora* or *Pseudomonas syringae* as a positive control.

4) **Protease** activity. To observe the hydrolysis of **casein** make a streak-plate on one half of **milk agar** medium plate. Use *B. subtilis* cells as a positive control. Incubate the plate at 30 °C for 1-7 days. Examine the formation of proteolysis zones around the inoculation line.

5) **Protease** activity. To observe the hydrolysis of **gelatin** make a stab culture into gelatin medium. The incubation must be for at least 2 weeks at 30 °C. Evaluate the results after keeping the test tubes on ice. In case of negative reaction, the medium in test tubes is solid, whereas in case of positive reaction, the surface or the whole medium is liquefied. Don't mix the test tubes during examination as the hydrolysis of gelatin progresses slowly from the surface of the medium to the bottom! Use cells of *Proteus vulgaris* as a positive control.

6) **Tryptophanase** activity is determined by inoculating the isolated strain into **tryptophan-rich medium** (LB-broth). Incubate for 1-2 days at 30 °C and prove indole with Kovacs reagent. **Carry the work out under fume hood!** Add 0.3 ml of reagent to the test tube, mix and indole is present if red alcohol layer forms on the aqueous layer. Use *E. coli* culture as a reference strain.

7) In **oxidase test**, cyt c oxidizes colorless p-phenylenediamine to **blue** indophenol during 10-60 s. Immerse the filter paper with oxidase test reagent and place some cells of the studied microbe on there with a sterile toothpick. Observe formation of blue color in case of positive reaction. The reagents are mixed immediately before the test! Commercial test strips which work on the same principle can also be used. Use *P. putida* culture as a reference strain.

8) **Catalase** activity is identified by dispersing the studied microbial cells in a drop of 3% **H_2O_2** on a microscope slide and formation of oxygen bubbles is observed.

9) **Arginine dihydrolase system** is identified by inoculating **LB+arginine liquid medium** with the studied microbial cells and covering the inoculation with sterile mineral oil. Observe the color change of the indicator dye (at first light red) to raspberry red due to pH increase. Use *P. putida* culture as a reference strain

10) **DNAse** is tested by streaking a **DNAse agar** with the studied microbial cells. The plate is examined after 24 h incubation for the formation of transparent zones around the inoculation lines. To make the zones more visible, 1-2 N HCl can be poured to the inoculation. Other possibility is to treat the plates with 0.1% toluidine blue (intact DNA is blue, hydrolyzed pink). Use *Serratia marcescens* culture as a reference strain.

3. DENITRIFICATION

Inoculate a high column of **Hiltay** medium (contains NO_3^- ions) with the studied microbe. Observe the alkalification of the medium and formation of gas (see chapter V). Don't mix the test tubes! Use *Pseudomonas fluorescens* culture as a reference strain

4. GROWTH AT DIFFERENT TEMPERATURES

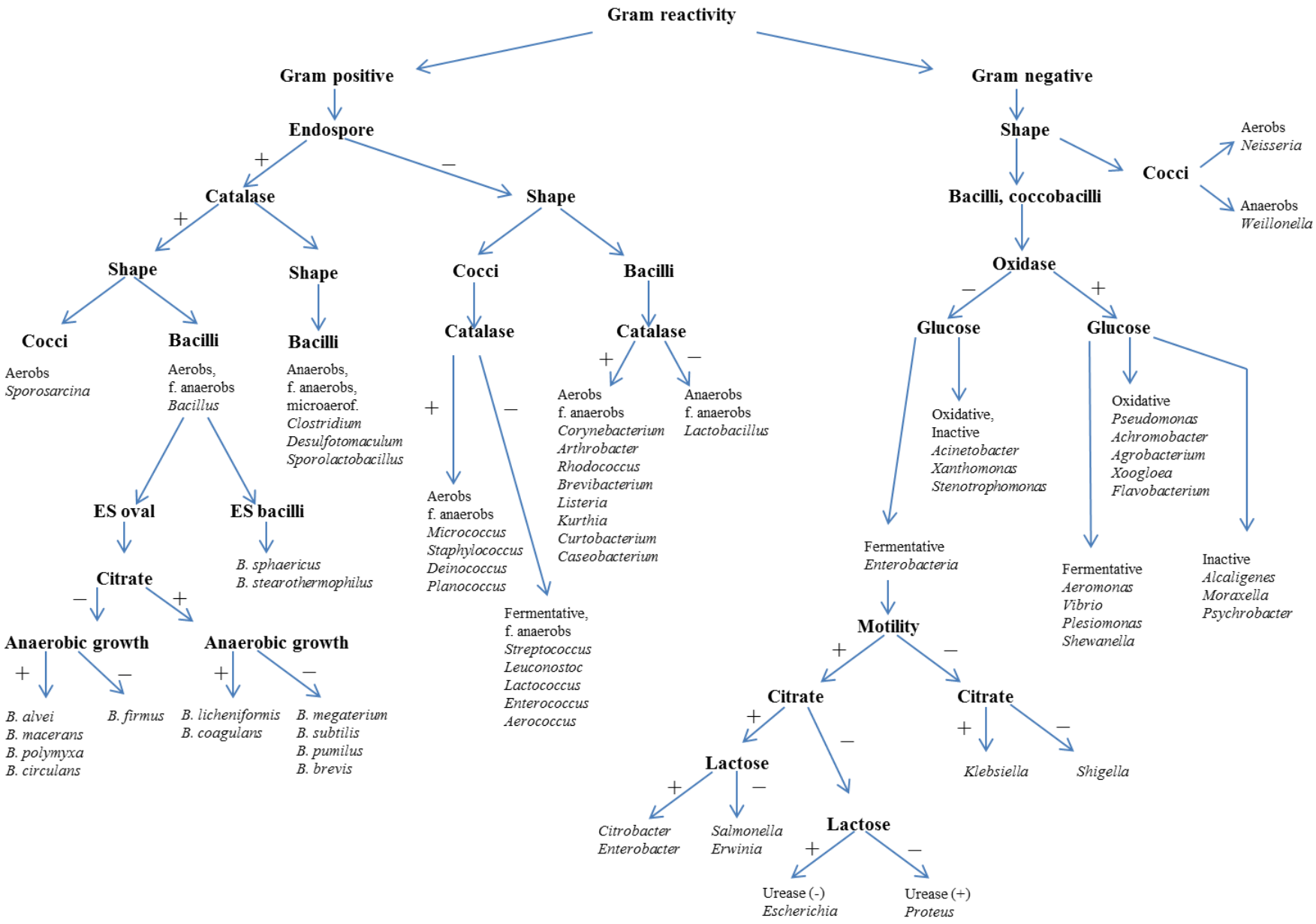
Inoculate **nutrient broth** with the isolated strains. Make sure that the medium is not turbid after inoculation. Incubate at different temperatures according to the suggestions made by the supervisor. The growth is evaluated by the formation of turbidity.

5. SALT TOLERANCE

Streak nutrient agar plates containing different concentrations of NaCl with the studied bacteria and incubate the marked plates at 30 °C. Choose the salt concentrations based on the suggestions of the supervisor. The growth is evaluated visually by the strength of the inoculation line.

D. IDENTIFICATION

1) Based on the phenotypic characteristics the unknown microbe can be positioned into a genus, using the dichotomic scheme below for principal mesophilic heterotrophs.
2) Including the information about 16S rRNA gene sequence (this will be provided by the supervisor), the strain should be identified as precisely as possible using the principles of polyphasic identification and *Bergey's Manual of Systematic Bacteriology* and other sources (e.g. typing a genus name obtained from the 16S rRNA gene comparisons to PubMed database search engine http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=search&db=PubMed). Fill out the table with the data of the closest reference strain and make the final conclusions about the identification of the unknown strain.


Gram reactivity
Gram positive
Endospore
+
Catalase
+
Shape
Cocci
Aerobs
Sporosarcina
Bacilli
Aerobs,
f. anaerobs
Bacillus
ES oval
Citrate
–
Anaerobic growth
+
B. alvei
B. macerans
B. polymyxa
B. circulans
–
B. firmus
+
Anaerobic growth
+
B. licheniformis
B. coagulans
–
B. megaterium
B. subtilis
B. pumilus
B. brevis
+
ES bacilli
B. sphaericus
B. stearothermophilus
Shape
Bacilli
Anaerobs,
f. anaerobs,
microaerof.
Clostridium
Desulfotomaculum
Sporolactobacillus
–
Shape
Cocci
Catalase
+
Aerobs
f. anaerobs
Micrococcus
Staphylococcus
Deinococcus
Planococcus
–
Fermentative,
f. anaerobs
Streptococcus
Leuconostoc
Lactococcus
Enterococcus
Aerococcus
Bacilli
Catalase
+
Aerobs
f. anaerobs
Corynebacterium
Arthrobacter
Rhodococcus
Brevibacterium
Listeria
Kurthia
Curtobacterium
Caseobacterium
–
Anaerobs
f. anaerobs
Lactobacillus
Gram negative
Shape
Cocci
Aerobs
Neisseria
Anaerobs
Weillonella
Bacilli, coccobacilli
Oxidase
–
Glucose
Fermentative
Enterobacteria
Motility
+
Citrate
+
Lactose
+
Citrobacter
Enterobacter
–
Salmonella
Erwinia
–
Lactose
+
Urease (-)
Escherichia
–
Urease (+)
Proteus
–
Citrate
+
Klebsiella
–
Shigella
–
Oxidative,
Inactive
Acinetobacter
Xanthomonas
Stenotrophomonas
+
Glucose
Fermentative
Aeromonas
Vibrio
Plesiomonas
Shewanella
Oxidative
Pseudomonas
Achromobacter
Agrobacterium
Xoogloea
Flavobacterium
Inactive
Alcaligenes
Moraxella
Psychrobacter

IDENTIFICATION CHART OF UNKNOWN BACTERIUM

Characteristic	Unknown strain	Reference strain
Colony:		
shape		
profile		
margins		
consistency		
color		
Fluorescent pigments on KingB agar		
Cell:		
Gram reactivity (mount and KOH test)		
Shape, aggregation		
Endospore		
Motility		
Utilization of sugars (growth/acid/gas) and other carbon sources:		
Glucose (O/F test)		
Sucrose		
D-fructose		
L-arabinose		
D-xylose		
D-mannitol		
citrate		
Fermentation type (Voges-Proskauer test)		
Denitrifification		
Ensymes:		
amylase (starch)		
arginine dihydrolase		
cyt c oxidase		
DNase		
catalase		
protease (casein)		
protease (gelatin)		
trypthophanase (indole test)		
urease		
β-galactosidase		
β-glucosidase (esculin)		
Growth at different temperatures and NaCl concentrations:		
37 °C		
42 °C		
Max% NaCl		

Conclusions: based on 16S rDNA sequence my strain belongs to the genus.....
Based on the phenotypic characteristics the closes species is.....
To conclude, my strain could belong to the species.....

IX ANTIBIOTIC SUSCEPTIBILITY OF BACTERIA

Three main features are characteristic to antibiotics: 1) they are produced by microorganisms; 2) they are either bacteriostatic or bactericidal; 3) they are selective and act at very low concentrations. Antibiotics are classified by their action bacteriostatic and bactericidal, depending on whether they inhibit microbial growth (erythromycin, tetracycline etc.) or kill microbes (penicillin, vancomycin, rifampin etc.). Most antibiotic producers are either molds (genus *Penicillum* – produces penicillin, griseofulvin), belong to the *Bacillus* genus (produce bacitracin, polymyxin) or actinomycetes (produce chloramphenicol, erythromycin, streptomycin, tetracycline). Antibiotics produced by actinomycetes are most commonly used in medicine (see Table 4). Antibiotics affect the cell wall, synthesis of nucleic acids or proteins, cytoplasmic membrane or inhibit specific enzymatic systems as substrate analogues and thus prevent the utilization of natural metabolite.

Table 4. Characteristics of important antibiotics

Antibiotic	**Chemical structure**	**Mechanism of action**	**Production**
Penicillin	β-lactam	cell wall	*Penicillium* spp
Oxacillin	β-lactam	cell wall	semisynthetic
Amoxicillin/ Clavulanic acid	β-lactam	cell wall synthesis/ β-lactamase inhib.	semisynthetic *Streptomyces* (*S.*) *clavuligerus*
Doxycyline	tetratcycline	protein synthesis	semisynthetic
Tetracycline	tetratcycline	protein synthesis	*Streptomyces* spp
Vankomycin	glycopeptide	cell wall	*S. orientalis*
Chloramphenicol	amphenicol	protein synthesis	synthetic, *S. venezuelae*
Erythromycin	macrolide	protein synthesis	*S. erythreus*
Streptomycin	aminoglycoside	protein synthesis	*S. griseus*
Gentamicin	aminoglycoside	protein synthesis	*Micromonospora* spp
Bacitratcin	polypeptide	cell membrane	*Bacillus licheniformis/subtilis*
Polymyxin B	polypeptide	cell membrane	*Bacillus polymyxa*
Nalidixic acid	quinolone	nucleic acids synthesis	synthetic
Ciprofloxacin	quinolone	nucleic acids synthesis	synthetic
Rifampin	rifampin	nucleic acids synthesis	semisynthetic *S. mediterranei*
Novobiocin	aminocoumarin	nucleic acids synthesis	*S. niveus*

Different antibiotics have different spectra of activity. Broad- (tetracyclin, chloramphenicol) and narrow-spectrum (natural penicillins, polymyxin) antibiotics are distinguished. For example, most GP and GN bacteria (*Neisseria, Legionella, Haemophilus*) are sensitive to erythromycin, but enterobacteria are resistant. Rifamycins are relatively new group of antibiotics produced by *Streptomyces*. It's semisynthetic derivate – rifampicin (rifampin) is used in tuberculosis treatment. **In general, GP bacteria are much more sensitive to antibiotics than GN bacteria.** This is explained by the differences in their cell wall structure and resistance plasmids that are rather widely spread among GN bacteria.

Microbial strains can be differentiated by their susceptibility to a certain antibiotic. In medical practice, it is necessary to determine the antibiotic susceptibility of the microbial strain for adequate therapy.

Antibiotic susceptibility is tested with **Kirby-Bauer method** which is based on using paper disks containing a defined concentration (μg or units) of antibiotic. Microbial culture with a standardized density (about 10^9 cells/ml) is applied on a medium plate as a lawn and then antibiotic disks are dispensed on the agar surface with a dispenser or sterile tweezers. After few hours of antibiotic diffusion to the medium in cold, the culture is incubated for about 20 hours at 30 ºC. The size of the growth free zones forming around the disks is not only dependent on the susceptibility of the microbe, but also on the antibiotics ability to diffuse to the medium. The criteria for evaluating the growth free zone diameters and antibiotic concentrations in the disks are standardized (see the table at the end of the practical works).

Disk method is also suitable for testing antibiotic susceptibility of microbes isolated from the environment. A disk with antibiotics is dispensed in the middle of the medium plate and kept in cold for few hours for the diffusion of the active compound. Then, radial inoculations are made on the plate with different microbial strains of certain density (inoculation line moving from the disk to the edge of the plate). The growth free zones forming around the disk show the antibiotic susceptibility of the microbe.

It is important to strictly follow that the right medium for testing antibiotic susceptibility is used, and that the thickness of the medium in the dish would be uniform (25 ml of medium in a dish with a diameter of 100 mm).

QUESTIONS

1. What properties characterize antibiotics?
2. Which microbes that produce antibiotics do you know?
3. What properties are classification of antibiotics into groups based on?
4. How is antibiotic susceptibility of a bacteria tested?
5. Why are gram-negative bacteria resistant to penicillin?

PRACTICAL WORKS AND RESULTS

In a group, determine **the susceptibility of test strains *E. coli, S. aureus*** or ***P. aeruginosa* to different antibiotics** by Kirby-Bauer method.
To study antibiotic (**AB**) sensitivity of the microbes use special media for testing antibiotics (see APPENDIX II, Mueller Hinton II Agar).

Protocol:

1) According to the suggestions by the supervisor make a spread-plate of ***E. coli, S. aureus*** or ***P. aeruginosa*** culture with a defined density and inoculum size of 0.1 ml*;*
2) divide the plate in mind to as many parts as there are discs to be applied. In the middle of each part place one disk immersed with a certain antibiotic (suggested by the supervisor);
3) place the plates into the fridge for 1 hour and then incubate for 24 h at 30 ºC.
4) Next day, observe the formation of growth-free zones around the antibiotic discs and evaluate the sensitivity of that microbe to different antibiotics. Fill the susceptibilities (inhibition zone diameter, mm) to the table below together with evaluation (R, I or S).

Antibiotic	**Disk concentration (µg or U)**	**Disk symbol**	**Diameter of inhibition zone (mm) ***			**Zone diameter (mm) and evaluation of susceptibility of test strains**					
						E. coli		***P. aeruginosa***		***S. aureus***	
			R	***I***	***S***	mm	Rating	mm	Rating	mm	Rating
Penicillin	10 U	P10	<12	12-21	>21						
			<21	21-28	>28						
			in case of *S. aureu*								
Oxacillin	1 µg	OX1	10	11-12	>13						
Amoxicillin+ Clavulanic acid	20 µg+10 µg	AMC30	<13	14-17	>18						
Carbenicillin	100 µg	CB-100	≤13	14-16	≥17						
			in case of *P. aeruginosa*								
			≤19	20-22	≥23						
			in case of *Enterobacteriaceae* ja *Acinetobacter*								
Doxycyline	30 µg	D	≤12	13-15	≥16						
Tetracycline	30 µg	TE30	<15	15-18	>18						
Vankomycin	30 µg	VA30	<10	10-11	>11						
Chloramphenicol	30 µg	C	<13	13-17	>17						
Erythromycin	15 µg	E15	<13	14-22	>23						
Gentamicin	10 µg	GM10	<12	13-14	>15						
Streptomycin	10 µg	S10	<12	12-14	>14						
Nalidixic acid	30 µg	NA	<14	14-18	>18						
Ciprofloxacin	5 µg	CIP5	<15	16-20	≥21						
Novobiocin	30 µg	NB30	<17	18-21	>22						
Rifampin	5 µg	RA5	≤16	17-19	>20						
Polymyxin B	300 U	PB300	<9	9-11	>11						

* Rating: R –resistant, I – intermediate, S – susceptible.

APPENDIX I: DYES AND REAGENTS

BARRITT's REAGENT
A: α-naphthol – dissolve 6 g in 96% ethanol – 100 ml;
B: KOH – 40%;
Prepare and use this reagent **only under the fume hood!**

HUCKER CRYSTAL VIOLET (ammonium oxalate crystal violet)
Prepared by mixing two solutions (A and B):
A – dissolve 2 g of crystal violet in 20 ml 95% ethanol;
B – dissolve 0.8 g of ammonium oxalate in 80 ml distilled water.
After mixing the day solution is left for 24 hours and then filtered

KOVACS' REAGENT
p-dimethylaminobenzaldehyde – 50 g;
Butanol – 750 ml;
Conc. HCl – 250 ml;
Prepare and use this reagent **only under the fume hood!**

CRYSTAL VIOLET CAPSULE DYE
Dissolve 1 g of crystal violet in 100 ml distilled water.

LUGOL SOLUTION
KJ – 2 g;
I_2 crystals – 1 g;
Add 5 ml of water to potassium iodide and then add iodine crystals. Add remaining water (295 ml) only when iodine has dissolved. **Store in a dark bottle and if solution has turned pale it should not be used!**

MALACHITE GREEN SOLUTION (also known as brilliant green or victoria green)
Dissolve 5 g of malachite green in 100 ml of distilled water.

METHYLENE BLUE SOLUTION
1% aqueous solution, filter before use.

NEUTRAL RED SOLUTION
1% aqueous solution, filter before use, store in a dark bottle.

NIGROSIN SOLUTION (Dorner's)
Nigrosin – 10 g;
Distilled water – 100 ml;
Heat for 30 min. Add 0.5 ml 40% formaldehyde (preservative) and filter the solution through a paper filter.

OXIDASE TEST REAGENT
α-naphthol – dissolve 30-40 mg in 96% ethanol – 2.5 ml;
Dimethyl-p-phenylenediamine dihydrochloride – 40-60 mg;
Distilled water 7.5 ml. Mix immediately before use!

ONPG (o-nitrophenyl-β-D-galactoside)
ONPG – 4 mg/ml in distilled water.

SAFRANIN
Dissolve 0.25 g of safranin in 10 ml of 95% ethanol and then add 90 ml of distilled water.

SUDAN BLACK B
Dissolve 0.25 g of sudan black in 100 ml of 70% ethanol.

TRIS – ACETATE BUFFER (TAE), 20x
Tris-(hydroxymethyl)-aminomethane – 121.1 g;
EDTA – 7.44 g;
Dissolve in 1000 ml of distilled water.
Adjust the pH to 8.3 with ~20 ml conc. acetic acid.

TOLUIDINE BLUE SOLUTION
1% solution.

VITRIOL OF COPPER SOLUTION
Dissolve 20 g of $CuSO_4 \times 5H_2O$ in 80 ml of distilled water.

FLAGELLA DYE
Solution I: 10 ml of 5% phenol solution in distilled water, 2 g of tannic acid, 10 ml of saturated $AlK(SO_4)_2$ solution in water.
Solution II: saturated crystal violet solution in ethanol (12 g of crystal violet dissolved in 100 ml of 96% ethanol).
The dye is prepared by mixing 1 part of solution II and 10 parts of solution I. The reagent is filtered before use and stored airtight in dark.

APPENDIX II: MEDIA

ANTIBIOTIC SUSCEPTIBILITY MEDIUM (Mueller Hinton II Agar)
Dissolve 38 g of powder concentrate in 1000 ml of distilled H_2O. Autoclave for 15 min at 121 ºC. Ingredients per 1000 ml of H_2O in g-s: meat extract – 2; CAA – 17.5; starch – 1.5; agar – 17. Medium pH 7.3.

ARGININE DIHYDROLASE MEDIUM
Luria-Bertani broth is the medium base (see below); 1% L-arginine, pH is adjusted to 6.8. 0.006% of indicator dye phenol red is added. Autoclave for 15 min at 112 ºC.

BASAL MEDIUM FOR CARBOHYDRATE UTILIZATION
Ingredients per 1000 ml of distilled H_2O in g-s: yeast extract – 1.0; peptone – 2.0; phenol red – 0.04; bromothymol blue – 0.02; agar – 2.5. Adjust pH to 7.1 (cherry red color). Sterilize for 15 min at 121 ºC. Different filter-sterilized carbohydrates are added aseptically to the medium before aliquoting with a final concentration of 1%.

BRILLIANT GREEN – BILE 2% BROTH (BGLB)
Dissolve 40 g of powder concentrate in 1000 ml of distilled H_2O. Distribute into test tubes fitted with inverted Durham tubes in 10 ml aliquots. The volume of medium can vary depending on the sample size. For 10 ml samples dissolve 80 g of powder and aliquot it by 10 ml-s into test tubes. Autoclave for 15 min at 112 ºC. Ingredients per 1000 ml of H_2O in g-s: peptone – 10.0; lactose – 10.0; bile – 20.0; brilliant green – 0.0133. Medium pH 7.2.

CHRISTENSEN MEDIUM
Ingredients per 1000 ml of distilled H_2O in g-s: peptone – 1.0; glucose – 1.0; NaCl – 5.0; Na_2HPO_4 – 1,2; KH_2PO_4 – 0.8; yeast extract – 0.1; phenol red – 0.012; agar -15. Medium pH must be between 6.8-6.9 (salmon pink color). Autoclave for 15 min at 112 ºC. **Filter-sterilized urea solution** (5 ml 40%) is added into sterile autoclaved and cooled medium prior aliquoting (final concentration 0.2%).

DNAse TESTING AGAR
Dissolve 39 g of power concentrate in 1000 ml of distilled H_2O. Ingredients per 1000 ml of distilled H_2O in g-s: tryptose – 20.0; DNA – 2; NaCl – 5.0; agar – 15.0. Autoclave for 15 min at 121 ºC.

ENDO MEDIUM
Dissolve 41.5 g of powder concentrate in 1000 ml of distilled H_2O. Ingredients per 1000 ml of distilled H_2O in g-s: peptone – 10.0; lactose – 10.0; K_2HPO_4 – 3.5; Na_2SO_3 – 2.5; alkaline fuchsine – 0.5, agar – 15. Medium pH is 7.5. The medium must be pale pink and is light sensitive.

ESCULIN HYDROLASE MEDIUM
Dissolve 16.5 g of Esculin Iron Agar powder concentrate (0.1% esculin, 0.05% ammonium iron(III) citrate, 1,5% agar) and 8.0 g of nutrient broth powder concentrate (ingredients are listed below) in 1000 ml of distilled H_2O. Autoclave for 15 min at 121 ºC.

GELATIN MEDIUM
Use nutrient broth powder concentrate prepared from meat broth and peptone as a base for the medium. Ingredients per 1000 ml of distilled H_2O in g-s: nutrient broth – 8.0; gelatin – 150.0. Autoclave for 15 min at 121ºC.

HILTAY MEDIUM
Ingredients per 1000 ml of distilled H_2O in g -s: KNO_3 – 2.0; asparagine – 1.0; Na- or K-citrate – 5.0; KH_2PO_4 – 2.0; $MgSO_4$ x $7H_2O$ – 2.0; $CaCl_2$ x $6H_2O$ – 0.2; $FeCl_3$ – traces; bromothymol blue – 0.08 g. Medium pH is adjusted to 6.8-7.0, indicated by moss green color of the medium. Sterilize for 15 min at 121 ºC.

HUGH – LEIFSON MEDIUM
Ingredient per 1000 ml of distilled H_2O in g-s: peptone – 2.0; NaCl – 5.0; K_2HPO_4 – 0.3; glucose – 10.0; bromothymol blue – 0.03; agar – 3.0. Adjust medium pH to 7.1 (green color). Sterilize for 15 min at 112 ºC.

KING B MEDIUM
Dissolve 38 g of powder concentrate in 1000 ml of distilled H_2O and add 10 ml of glycerol. Ingredients per 1000 ml of distilled H_2O in g-s: peptone – 20.0; glycerol – 10.0; $MgSO_4$ x $7H_2O$ – 1.5; KH_2PO_4 – 1.5; agar – 15. Adjust medium pH to 7.2. Autoclave for 15 min at 121 ºC.

LAURYL SULFATE BROTH with MUG (4-methylumbelliferyl-β-D-glucuronide)
Dissolve 35.7 g of powder concentrate in 1000 ml of distilled H_2O. Autoclave for 15 min at 112 ºC and cool as quickly as possible. If agar plates are prepared, add 15.0 g of agar. Ingredients per 1000 ml of H_2O in g-s: Na-lauryl sulfate – 0.1; tryptone – 20.0; lactose – 5.0; K_2HPO_4 – 2.75; KH_2PO_4 – 2.75; NaCl – 5; MUG – 0.05. Adjust pH to 6.8. After incubation with microbes, measure fluorescence in the longer wavelength region of UV (at 366 nm).

LURIA - BERTANI (LB) AGAR MEDIUM
Ingredients per 1000 ml of distilled H_2O in g-s: tryptone – 10.0; yeast extract – 5.0; NaCl – 5.0; agar - 15. Autoclave for 15 min at 121 ºC.

METHYL RED – VOGES-PROSKAUER MEDIUM (MR-VP)
Ingredients per 1000 ml of distilled H_2O in g-s: peptone – 7.0; K_2HPO_4 – 5.0; glucose – 5.0. Adjust medium pH to 6.9. Sterilize for 15 min at 112 ºC.

400X CONCENTRATE OF MICROELEMENTS
Ingredients per 1000 ml of distilled H_2O in g-s: MgO – 10.75; $CaCO_3$ – 2.0; c. HCl –51.3 ml; $FeSO_4$ x $7H_2O$ – 4.5; $ZnSO_4$ x $7H_2O$ – 1.44; $MnSO_4$ x $4H_2O$ – 1.12; $CuSO_4$ x $5H_2O$ – 0.25; $CoSO_4$ x $7H_2O$ – 0.28; H_3BO_3 – 0.06. Sterilize for 15 min at 121 ºC.

10X CONCENTRATE OF MINERALS MIX M9
Ingredients per 1000 ml of distilled H_2O per g-s: Na_2HPO_4 – 70.0; KH_2PO_4 – 30.0; NaCl – 5.0; NH_4Cl – 10.0. Adjust the pH to 6.8. Sterilize for 15 min at 121 ºC.

MILK COMPLEX MEDIUM
Many microbes need various growth factors in their growth environment. Thus it is practical to use the complex medium version of milk medium: sterilize (15 min 121 ºC) 900 ml of tryptone-soy agar (composition provided later) and add separately autoclaved (15 min 112 ºC)

100 ml of fat-free milk. If needed, 0.1% yeast extract can be added to tryptone-soy agar before autoclaving.

NUTRIENT AGAR
Dissolve 23.0 g of powder concentrate in 1000 ml of distilled H_2O. Autoclave for 15 min at 121 ºC. Ingredients per 1000 ml of H_2O in g-s: meat extract – 3.0; peptone – 5.0; agar – 15.0. Medium pH is 6.8.

NUTRIENT AGAR WITH GLUCOSE
Ingredients per 1000 ml of distilled H_2O in g-s: nutrient agar – 23.0; glucose – 5.0. Autoclave for 15 min at 112 ºC.

NUTRIENT BROTH
Dissolve 8.0 g of powder concentrate in 1000 ml of distilled H_2O. Autoclave for 15 min at 121 ºC. The composition is similar to nutrient agar, but no agar is added here.

R2A AGAR
Dissolve 18.2 g of powder concentrate in 1000 ml of distilled H_2O. Sterilize for 15 min at 121 ºC. Ingredients per 1000 ml of H2O in g-s: yeast extract – 0.5; peptone – 0.5; CAA – 0.5; glucose – 0.5; soluble starch – 0.5; Na-pyruvate – 0.3; K_2HPO_4 – 0.3; $MgSO_4$ – 0.05; agar – 15. Adjust medium pH to 7.2 and autoclave for 15 min at 121 °C.

SIMMONS CITRATE AGAR
Ingredients per 1000 ml of distilled H_2O in g-s: Na-citrate – 2.0; NaCl – 5.0; $MgSO_4$ – 0.2; $NH_4H_2PO_4$ –1.0; K_2HPO_4 – 1.0; agar – 15. Adjust medium pH to 6.9, so the indicator dye bromothymol blue (0.08 g) turns moss green. Sterilize for 15 min at 121 ºC.
Powder concentrate can be used for preparing this medium and if needed, agar can be added to the final concentration of 1.5%.

STARCH MEDIUM
Ingredients per 1000 ml of distilled H_2O in g-s: tryptone – 10.0; yeast extract – 10; K_2HPO_4 – 5.0; soluble starch – 3.0; agar – 15.0. Dissolve starch in small amount of distilled water before adding to the medium. Autoclave for 15 min at 121ºC.

TRYPTONE-SOY BROTH (TSB)
Dissolve 30.0 g of powder concentrate in 1000 ml of distilled H_2O. Autoclave for 15 min at 121 ºC. Ingredients per 1000 ml of H_2O in g-s: tryptone – 17.0; soya peptone – 3.0; glucose – 2.5; NaCl – 5.0; K_2HPO_4 – 2.5. Medium pH is 7.3.

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