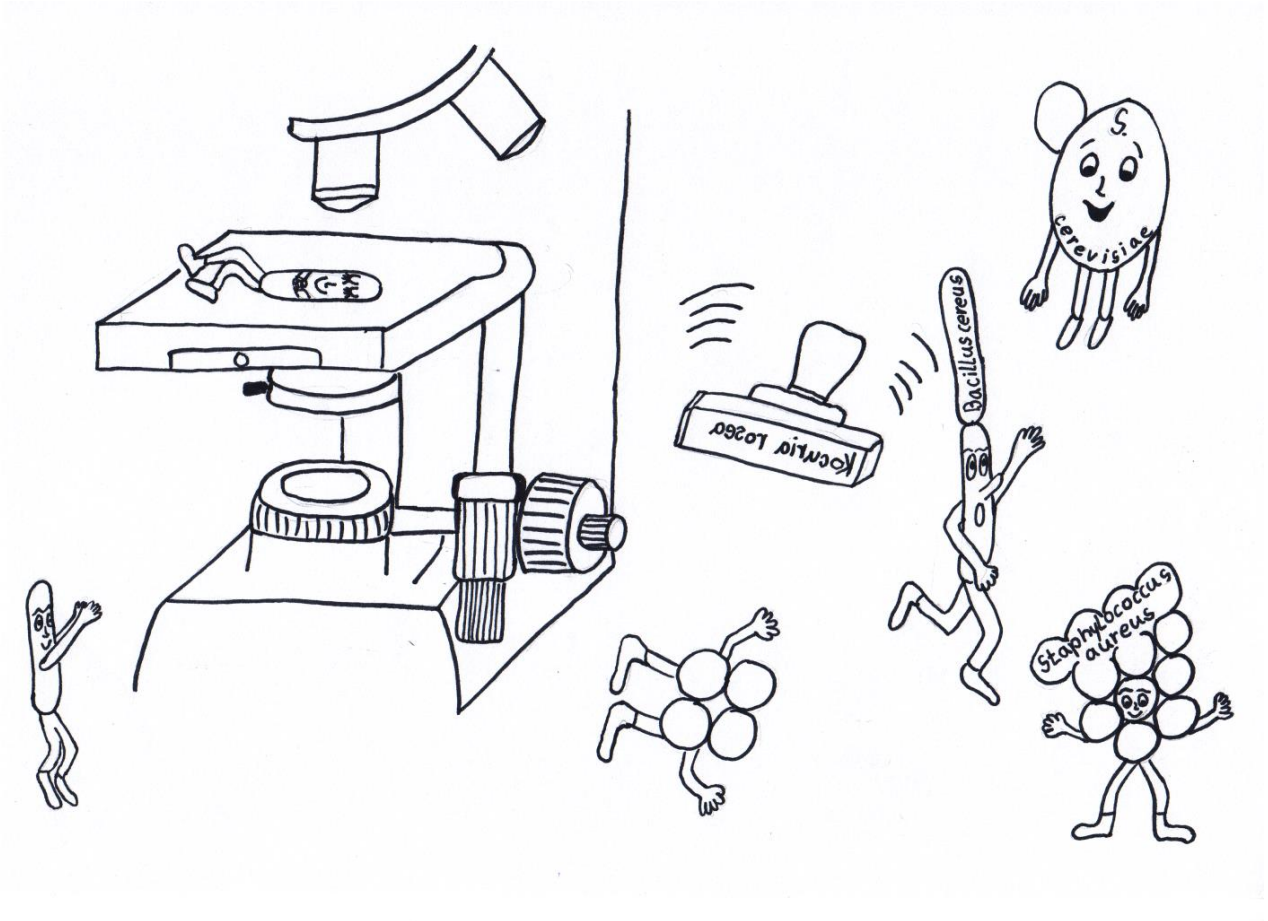
SCRIPT TO THE PRACTICE FROM GENERAL MICROBIOLOGY, CYTOLOGY AND MORPHOLOGY OF BACTERIA



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Preface

Bakterie, viry a kvasinky nepatří do stejné taxonomické rodinky.
Se všemi se seznámíme a možná se i něco přiučíme.
V praktiku je nutné asepticky pracovat, abychom se mohli z hezkých výsledků radovat.
Při plotnové metodě by měly být výsledky ve shodě.
Míchat vzorek se vždy musí, každý si to prakticky zkusí.
Inhibiční zóny vznikají, když citlivé bakterie se s antibiotiky setkají.
Při vitálním barvení docházíme k zjištění,
barvu nepřijme buňka živá, protože je její membrána funkční a celá.
Pokud s fágy pracujeme, pak i bakterie potřebujeme,
aby se fágy mohly pomnožit a fázový lyzát či plaky vytvořit.
Buď pozitivní modré či negativní červené, jsou buňky po "Gramovi" zbarvené.
Při rozboru vody mají bakterie hody,
kultivujeme je na selektivním médiu, někdy hokejkuju a někdy vzorek médiem zaliju.
Jde nám o enterokoky a bakterie koliformní, ve vodě není vhodný jejich výskyt enormní.
Postupná identifikace a testy základní jsou pro správné určení vždycky to zásadní.

Authors

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We would like to thank to Andrea Vávrová for helping with preparation of background of practices.

Content

Principles of safety work in microbiological laboratory

1. Entry to the laboratory is allowed only for person having the practices.
2. Do the experiments only described by the practice content in laboratory.
3. Eating, drinking and smoking is forbidden in laboratory.
4. It is necessary to wear laboratory coat and protective overshoes.
5. Opening of windows is forbidden in laboratory. Aerating is established by the air-conditioning.
6. Read the laboratory practice content before practice.
7. It is necessary to disinfect the working area, wash and disinfect your hand before and after practice.
8. Do not put the personal objects on working surface of table. Things could be easily contaminated by microorganisms. Take off clothes, bags in changing room.
9. Work carefully. Prevent contamination of material and accidental staining of work area and yourself from bacterial cultures.
10. Do not touch unnecessarily the face, do not wear cosmetics in laboratory, do not manipulate with contact lenses in laboratory.
11. Use single protective gloves during the bacteria staining, if it is possible work in digestor. Protective gloves is not needed to use during manipulation with microorganisms, if you would feel more safely, wear it.
12. Use the gas burner only when you are in the same room.
13. Put away the used glass and rests of bacterial cultures on marked places. Do not pour cultures in the normal waste! All contaminated material is disinfected and sterilised before liquidation and washing (also the broken glass), eventually is placed in special basket for dangerous waste (i.e. cellulose used to liquidation of poured culture).
14. If accidental tarnish of skin or injury happens, let it know to tutor/teacher immediately. It is necessary to treat the skin suitable disinfection agent (ajatin, Spitaderm) to avoid the infection.
15. The same priniples as in point 14 are valid for contamination or working area or working clothes.
16. Inform yourself about the right procedure in any case of your diffidence at your tutor/teacher.
17. Sign all media and cultures in tubes, flasks and Petri dishes by the name of medium and culture, your name and work group. Label dishes on the bottom! Use felt-tips on glass.

18. Record carefully all working procedures, especially used bacterial cultures, amount/volumes of solution and dilution procedures.

19. Put the used aids on marked place after working, clean and disinfect the working area.

20. Wash and disinfect your hands properly before laboratory leaving. If you need a short pause during the practice, wash and disinfect your hands properly before laboratory leaving.

PRACTICE FROM GENERAL MICROBIOLOGY

Preparation of nutrient media, cultivation of microorganisms, aseptic work

Aim of practice

Preparation and sterilization of nutrient media (agar on Petri dishes, slant agar and broth). Principles of aseptic work in laboratory.

Introdcution

Principles of microbiological media preparation

It is necessary to work with sterile aids in sterile environment, speed is more important htan volume accuracy (aseptic work). Singe the neck of flasks and tubes. Do not talk during working. Open the vessels with sterile media at least.

Cultivation

Cultivation of microbes is the basic procedure of their direct. Character of bacterial growth is important identification marker; disadvantage is time of cultivation (i.e. *Mycobacterium tuberculosis* grows for 9 weks, most of bacteria for 24-48 hours). Microorganisms (bacteria, yeasts) are cultivated on sterile nutrient media that accomplish all demands for nutrition, have optimum pH, osmotic ratio, redoxpotential, sufficient amout of water and nutrients: **source of energy** (organothrophic – organic compounds; photothrophic - light; litothrophic - inorganic compounds), **source of carbon** (heterothrophic – organic compounds; autothrophic - CO_2), **source of nitrogen** (ions of ammonia, nitrates, aminoacids, proteins or jejich their hydrolysates) and **biogenic elements** (inorganic salts).

Nutrient media

Media are divided in two groups according to composition: **synthetic (defined)** with accurately defined composition (organic solutions, source of carbon is mostly glucose, source of nitrogen $(NH_4)_2SO_4$ or NH_4Cl, pure aminoacids, vitamins, growth factora) and **natural** (complex) with nutrient broth without chemical definition. Nattural media comprises component from acidic hydrolysis of casein, gelatine or enzymatic hydrolysis of meat (pepsin, trypsin, pankreatin).

According to conzistence: **liquid** (milk, meat-peptone broth, sugar solutions, malt), **semi-solid, solid**. Advantage of liquid media is easy access to water and nutrients,

microorganism grow better. Disadvantage is grow in form of turbidity, sediment – the purity of culture or mixture of strains could not be determinated.

Mostly agar (mixture of polysaccharides from marine algae, does not serve as source of nutrient), gelatine (lower temperature of melting, circa 35 °C) or silica gels are added to broth for solidification. Advantage of cultivation on solid media in Petri dish is the possibility to observe isolated colonies (clones of one cell), isolated strains. Colony of bacterial strain is characteristic and taxonomic macroscopic marker.

Universal media satisfy a broad spectrum of organisms with its composition (i.e. meat-peptone bujon, malt agar). **Selective media** prefere growth of one species/strain or target group of organisms by their composition, th growth of another strains is inhibited (i.e. Ashby agar – medium with no source of nitrogen, only organisms capable to fix air nitrogen). Selective media contain inhibition compound or some basic compound is lacking that prefere and isolate target species and genere. **Selective-diagnostic media** inhibite growth of most microorganisms and enable to grow only small group of organisms. Characteristic growth is manifest by change of media or colony colour (i.e. Endo agar).

Examples of cultivation meida

Meat-peptone agar (MPA) – meat extract, peptone, salts, agar, base for another media.

Blood agar (KA) – addition of 5-10% defibrined animal blood to MPA, the most used medium, most of bacteria grow on it, hemolysis could be readed – total or partly clarifying of medium.

Endo agar (EA) – selective-diagnostic for intestinal bacteria (family *Enterobacteriaceae*), contains lactose. Indicator of fermentation is basic fuchsine with sodium sulphite.

XLD agar - for pathogenic intestinal bacteria (*Shigella, Salmonella*), contains lactose. Laktose positive bacteria ale yellow, H_2S formation produces black center of colonies.

Sabouraud medium – for yeast and moulds, contains glucose or maltose, pH 5,0.

Fortner medium - for anaerobes (reducing substances).

Löwenstein-Jensen medium – solid medium for mycobacteria, contains eggs, glycerin, starch, malachite green.

Slanetz-Bartley agar (SB) – selective-diagnostic, for genus *Enterococcus,* nutrients poor, colonies of enterococci have violet-brown colour.

Wilson-Blair medium – selective for genus *Salmonella* (black metallic lustre colonies).

Clauberg medium - diagnostic for *Corynebacterium diphtheriae* (black colonies with metallis lustre).

Chocolate agar – blood is added to hot agar (80 °C), serves for cultivation of harf of cultivating microbes.
Mueller-Hinton agar – for antibiotics testing and for primary isolation of neisseria.

Storage of media

Media are stored packed in foil in fridge to avoid drying, with some exceptions. Fresh media have to be dried before innoculation of bacteria, no wet surface.

Disinfection, sterilization, decontamination

Elimination of microorganism from environment (decontamination) could be quaranteed by many ways. Cleaning, washing and ironing decrease the occurence of microorganism up to 90%. Effectivity of disinfection or sterilization is increased in this way.
Disinfection is defined as destroying and restraining of vegetative pathogenic cells from objects by usage of chemical or physical compounds in environment (water, air) and in infected material. The aim of disinfection is to make objects noninfect. Effectivity of disinfection is depend on microbial resistance against disifcent agents with bactericidal effect.
Antiseption is restraining of pathogenic germ in live tissue, injuries, on mucosa and onskin by using of antiseptics.
Aseption is the set of rules leading to minimum number of microorganisms in environment.
Sterilization is destroying of all live microorganisms including highly resistant bacterial spores by physical or chemical procedures.
Sterilization by satured water vapour under pressure (autoclave) is performed mostly at 100 kPa at 120 °C for 20-30 minutes. This type of sterilization enables to destroy all form of microorganisms. Autoclave is pressure sterilizator in that could be sterilize various solutions, metal medicinal instruments, rubber material. Attention have to be devoted by possible hydrolysis of saccharides and damaging of thermolabile compounds.

Instruments and microorganisms

commercial meat-peptone medium (MPB – meat peptone broth)
agar, distilled water
sterile Petri dishes
glass biological tubes, Erlenmeyer flasks

Methods

- Work is taken place in pairs. Sign your tubes and dishes from below.
- Weigh 2,6 g of commercial meat-peptone-broth into the Erlenmeyer flask.
- Fill into 200 ml with distilled water, measure pH (pH paper, eventually adjust) and mix properly.
- Pipette 5 ml of solution into two tubes, close with cotton-wool or metal plug. Tubes with broth are now prepared to sterilisation.
- Add 3,6 g of bacteriological agar to the rest of solution and mix. Warm up the medium until the agar is mushy (in autoclave or in microwave oven), pipette 5 ml of solution into two tubes, close with cotton-wool or metal plug. Tubes are now prepared to sterilisation - slant agar.
- Close the rest of medium in flask with plug and place it together with tubes in autoclave. Sterilization takes plase for 20 minuts (0,15 MPa, 121 °C).
- The medium is sterile after sterilization, it is necessary to maintain the principles of aseptic work (singe the necks of flasks and tubes)!
- Pour the sterile medium from flask into the strile Petri dishes. Pour into the dish circa 20 ml of medium (approximately 4-5 mm of medium in height). Open the dishes at minimu, do not talk during the pouring (fig. 1A). Turn the dishes upside down after solidification.
- Place the tubes with sterile agar in slant position before solidification and let it solidificate (fig. 1B).
- Assess potential contamination after a few days.

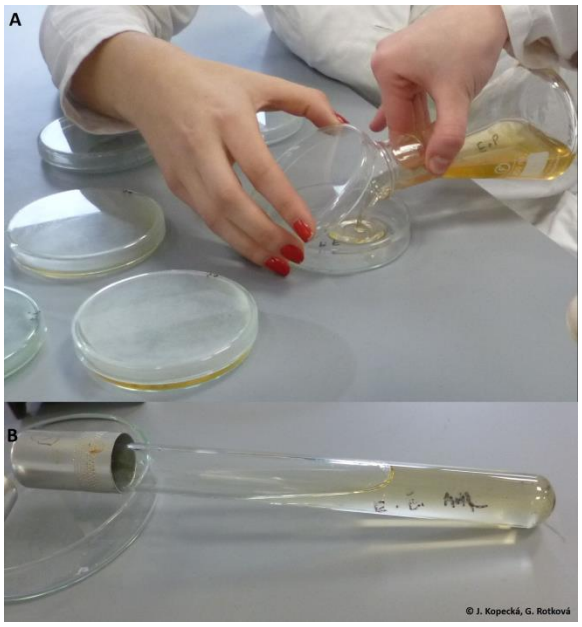


Fig. 1. Preparation of nutrient agars.

Conclusion

Prepared media will be used in the next practice for innoculation and additional macroscopically observing of microbial cultures. Did you keep the principles of aseptic work? Did contamination grow?

Additional information

Votava M., Kultivační půdy v lékařské mikrobiologii. 2000, Nakladatelství Hortus, Brno, ISBN 80-238-5058-X.

Control questions

1. How was the sterility of work guaranteed?
2. Which advantage has slant agar compared to agar in Petri dish?
3. Why is the character of colony growth evaluated on Petri dish (not in broth)?
4. Differences between synthetic and natural media.
5. Write examples ans composition of solidification compounds of cultivating media in microbiology.
6. Which aids and procedures guaratee the aseptic work in practice?
7. What is the purpose of pasteurisation?
8. How we can get solid medium from broth?
9. What material is sterilised hot-air dryer and in autoclave?

10. What is the functon of cultivation media?
11. What is the difference between antisepsis and aseptic work?
12. Are the bacterial spores inactivated by the pasteurisation?
13. What is the sterilisation; write some examples.
14. Write some examples for securing of aseptic work on laboratory table in microbiological practice without flowbox.
15. How we can enrich the cultivation medium?
16. What is disinfection?
17. What is sterilization?

Methods of sterile work, innoculation and storage of microorganisms

Aim of practice

Learning of principles aseptic work and use the principles by working with microorganisms. Innoculation of microorganisms by various techniques (broth and agar). Isolation of single colonies by streak plate.

Introduction

Microorganisms cultivated in laboratory on nutrient media are designated as **cultures**. Culture of one single species/strain is **pure culture**. **Mixed cultures** contain several species/strains/genera (i.e. isolates from natural environment). **Technical cultures** are used for esearch or industrila purposes (sewage disposal plants, bacterial filters, bioreactors), could be as pure culture (brewing yeasts) asi mixed cultures (lactic acid bacteria for yoghurts production).

Cultures are transferred (= innoculated) on fresh medium from liquid or solid medium to: recovery, innoculate diagnostical medium, isolate colonies, determinate physiologic and morphologic characteristic. Character of growth and cultivation conditions of individual strains in laboratory (optimum conditions – pure culture, enough of nutrients) are always different from growth in natural environment (competitions about nutrients, adaptation, presence of antibiotics and metabolites of others strains). Many bacterial strains in noncultivable.

Isolation of bacterial strain

Selective media are mostly used to isolate the pure. **Method of streak plate** is used to strain isolation on selective or nonselective (universal) medium on Petri dish**.** Various morphological types of growing colonies could be differentiated and isolated by subsequent streak plate (Innoculation of cells from one single colony). **Streak plate** is method of subsequent dilution of original sample to grow single colonies. Colony of microorganisms is the clone from one single cell. Bacteriologic loop is singed after single step during innoculation, cells are killed by flame and the lower amount of cells is smeared in subsequent step. In the last space of steak plate grow the single colonies in that characteristic profile, shape, colour, margins could be evaluated.

Cultivation

Cultures in liquid medium could be cultivated **continuously** (higher volumes with industrial strains kmeny). One example is **chemostat** where the growth is controled by concentration of nutrients that are added by inflow of fresh medium. Cultivation in medium without any inflow of nutrients is called **static**. Static cultivation could be **submerged (stirred/shaking)** or **aerated.**

Appearance of culture affects used medium, type of cultivation, age of culture. Same microorganism could show on various agars different morphological characteristics (pigmentation, size of colonies).

Cultivation conditions shloud be performed according to culture catalog, in our case pure cultures and catalog from Czech Collection of Microorganisms (http://sci.muni.cz/ccm/) – recommended defined media, temperature and cultivation conditions. Conditions of cultivation closed to original environment conditions are suitable for **natural isolates** (salts concentration, nutrients, temperature).

Growth curve (fig. 2) is graphic expression of relationship of cell number and time of static cultivation, composes from several phases:

1. **Lag phase** – adaptation and slow growth, activation of suitable enzymes, organisation of metabolism, presence of adaptive enzymes, high amount of RNA (increased enzymes synthesis), nonadapted cells are dying.
2. **Phase of accelerated growth** – point between lag and log phase; all enzymes are synthetised, high speed of growth.
3. **Log phase** (logarithmic, exponencial) – intensive growth and metabolism, length depend on concentration of limited nutrient, every cell is divided by constant maximum speed. Parameters are used for comparing experiments from this part of growth curve. Cells could be well characterised, growth character is always evaluated in log phase (dry and wet mass, amount of metabolites, assesment of DNA, RNA weight).
4. **Phase of reduced growth** – intensity of metabolism is decreased, accumulation of metabolites
5. **Stationary phase** – speed of reproduction is reduced, number of new cells equals to number of dead cells, depletion of nutrients
6. **Dying phase** – medium is consumed, cells reduce their own storage compounds, acidification of environment

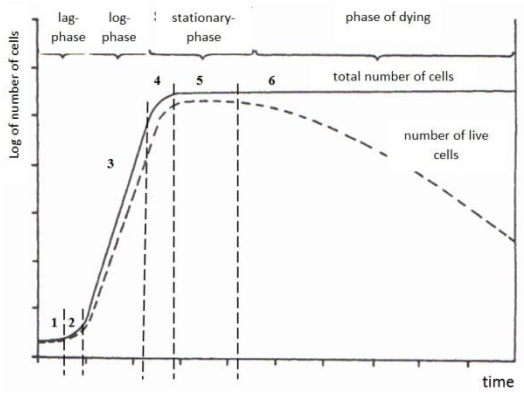


Fig. 2. Growth curve. Full line – total number of microorganisms (dead and live); dashed line – number of live microorganism (Greenwood et al., 1999, adjusted).

Diauxie is subsequent utilisation of two substrates. At first, the simple source, i.e. glucose, is utilised, after that more complicated substrate is utilised, i.e. lactose. Growth curve has two peaks in case of two substrates.

Temperature

Three basic group of microorganisms are differentiated according to optimum temperature: **psychrophilic,** growth optimum under 20 °C (oceans, caves, fridge – i.e. pseudomonades, aeromonades, listeria); **mezophilic,** growth optimum between 20 - 40 °C (most of bacterial species; parasitic microorganisms); **termophilic,** growth optimum over 55 °C (extremely termophilic grow at about 100 °C).

Relationship to oxygen

Bacterial strains cultivated on air are called **aerobic**. Aerobic cultivation is performed by smear of cell and cultivation on agar in Petri dish or in tube on slant agar or in low amount of liquid medium (about 5 ml). Higher volumes have to be aerated by oxygen (aeration, submerged cultivation). **Optional anaerobes** are capable grow as in aerobic as in anaerobic environment. They could swith to energy-profitable aerobic metabolism n oxygen environment. They grow in whole volume of liquid medium. **Anaerobic organisms** occurs in environment with no or very low oxygen concentration. Anaerobiosis was defined by Louis Pasteur. Anaerobic microorganisms are divided according to sensitivity to molecular oxygen: **strictly (obligatory) anaerobic microorganisms**, **aerotolerant microorganisms** grow in low oxygen concentration; **microaerophilic microorganisms** require certain low percent of oxygen.

Anaerobic or microaerophilic cultivation is performed by stab in agar or by innoculation in high layer of liquid. It is necessary to decrease the oxidation-reduction potential by addition of reducing compounds (ascorbic acid, thioglycolate, thiosulphate) or to use s.c. anaerostate and mixture of chemicals (iron powder, tartaric or citric acid) that release hydrogen in presence of catalyst (Pt, Pd) and react with oxygen.

Storage of microorganisms

Type of storage is choosed according to its length:

- on Petri dish at 4 °C, low-term, re-innoculation (i.e. lactococci after one week, bacilli after 2-3 months)
- stab in slant agar, months
- slant agar at 4 °C, weeks; at room temperature or in thermostate at 25 °C for only days
- on porous materials - gelatine discs, beads, long-term
- under sterile mineral oil (fungi, bacteria)
- **lyophylized** (lyophylization = freezing of water in vacuum), some microorganisms can not be lyophylized (fungi), moderately decreasing of viability, lyophylized cultures are ready to immediately innoculation and transfer, easy manipulation
- freezed at – 70 °C (i.e. in glycerol) in small volumes, months, years
- boxes with solid CO_2, dry ice **(- 78 °C)**
- cryogenic freezers (- 150 °C)
- **cryopreservation** – freezed cultures at very low temperatures, i.e. in liquid nitrogen (- 196 °C) or in another gases (He, Cr, H), very long-term. Usage of cryoprotectants in medium – dimethylsulfoxide, glycerol.

Characteristics of microorganisms used in practice

Escherichia coli (family *Enterobacteriaceae*): gramnegative stright rods, single or pairs. Peritrichous or immobile, mezophilic, optional anaerobic. Commensal of colon, synthesis of vitamines A, B, K. Outside colon could be pathogenic. It serves as model for genetic ingeneering, produces various compounds (i.e. insulin).

Pathogenic strains are characterised and identified serologically (somatic, capsular, flagella antigens): enterotoxigenic (ETEC) – traveler diarrhoea, endemic occurence; enteropathogenic (EPEC) – postneonatal diarrhoea, alteration of intestinal epithel; enteroinvasive (EIEC); enterohemorrhae (EHEC) - hemorrhae (bleeding to organs in digestive tract).

Serratia marcescens *(*family *Enterobacteriaceae)*: gramnegative straight rods, optional anaerobic, mezophilic, red pigment formation. Occurence in soil, water, on plants, opportune human pathogen – nosocomial infection of urinary and respiratory tract.

Pseudomonas *(*family *Pseudomonadaceae)*: gramnegative straight or slighty curved rods, one or more polar flagella. Optimum temperature between 25-30 °C, aerobic. Fenasine exopigments pyocyanin and fluorescin (pyoveridin) formation – yellow or bluegreen colour. Occurence in environment, food (eggs, fish, milk), clinical isolates. Significant human, animal and plants pathogens, nosocomial infections; factor of virulence if biofilm formation with high level of resistance on tissue surface or objects due to polysaccharides formation (*P. aeruginosa* or *P. fluorescens).* Broad spectrum of metabolic pathways - geochemical cycles, biodegradation, bioremediation (degradation of toluen), biocontrol agents.

P. putida – bioremediation; *P. fluorescens* – fluorescein production

Kocuria rosea *(*family *Micrococcaceae)*: grampositive cocci, pairs, tetrads or clumps. Aerobic, mezophilic, pink and red pigments formation. Occurence in soil, water, skin of vertebrates. Genus is named after meaningful microbiologist RNDr. Miloš Kocur.

Micrococcus luteus *(*family *Micrococcaceae):* grampositive cocci, pairs, tetrads or clumps, aerobic. Occurence on skin of vertebrates, food, soil, air and water. Yellow pigment production.

Staphylococcus aureus (family *Staphylococcaceae*): grampositive cocci, single, pairs or irregular clumps. White or creamy pigment. Occurence on skin and mucosa of warm-blooded vertebrates, food, environment. Soma strains are pathogenic, toxins production.

Bacillus (family *Bacillaceae*): grampositive straight rods, pairs, chains, mobile, aerobic or optional anaerobic. Occurence in soil, water, food. Some strains are pathogenic for human or insects. Toxins production. Oval or rounds endospores located terminal, subterminal, paracentral or central, spores could be used as biopesticides (*B. thuringiensis*). Shape, size and location of spore is characteristic marker for identification.

B. cereus – environment, cause gastroenteritis, alimentar intoxication

B. subtilis – environment, isolated from food poisoning

B. mycoides – environment, rhisoid growth

B. sphaericus - soil, water sediments, food

B. thuringiensis – pathogenic for insects, toxin production (parasporal body)

Saccharomyces cerevisiae: eukaryotes, mezophilic, optional anaerobic yeast. Ovoid cell bigger than bacterial cell. Cell wall does not contain peptidoglycan. Occurence in environment and in industry. First sequenced eukaryotic organism. Food production (beer,

wine, breads), various compounds production (i.e. insulin, recombinant vaccine HBsAg), model organism.

Instruments and microorganisms

Dishes with MPA „meat-pepton-agar“ (MEA „malt-extract-agar“ for *S. cerevisiae*)

Tubes with slant agar MPA (MEA for *S. cerevisiae*)

Tubes with broth MPB (MEB for *cerevisiae*)

Innoculation loops and needles, thermostat, gas burner

Escherichia coli CCM 3954

Pseudomonas putida, P. fluorescens

Serratia marcescens CCM 303

Kocuria rosea CCM 839

Micrococcus luteus CCM 169

Bacillus cereus CCM 2010

Staphylococcus aureus SA 812

Saccharomyces cerevisiae

Methods

No wild microorganisms from air, aids, human microflora must not get into the microbial culture or media – we have to maintain the principles of **aseptic work**. We always work in closed room, with washed hands, on disinfected table, near to the gas burner, at maximum speed. The necks of flask and plugs is necessary to singe in flame before and after working. **We never put the plugs on the table**, we always hold the plugs with ring-finger and little-finger (fig. 3). We let the vessels with cultures opened only for short time period near to the flame.

Fig. 3. Aseptic work – innoculation of microorganisms.

We cultivate the microorganisms **static, aerobic-cultivation on agars** (dishes and slant agars) **and in broth.** Strains are cultivated on recommended media in thermostat at optimum temperature of target culture. We cultivate always the cultures on Petri dishes upside down on grounds of formation of condensation water, that could drop on the surface of medium and mix the culture on dish. Medium is dried slowery also.

Everybody works independently in this practice.
Sign all tubes and Petri dishes with felt-tip (species, date, initials).

Everybody innoculate:

- 1 strain into the broth
- 1 strain on slant agar
- 2 or 4 various strains on 1 dish (2 or 4 „snakes“)
- 1 strain on Petri dish – streak plate
- Mixture of 2 strains on Petri dish – streak plate – the aim is isolation of 2 types colonies (innoculation of mixture of various pigmented strains or grampositive and gramnegative cultures)

Innoculation on slant agar

- Singe the innoculation loop and let it cool.
- In the left hand, take both tubes with slant agar, pull the plug of tubes with culture with little-finger of right hand (fig. 3).

- Singe the neck of tube.
- Put the sterile loop (singed and cooled) into the tube with culture, get a little amount of culture. Pull out the loop, singe the neck of tube, plug and close the tube.
- Pull the plug of sterile tube with medium with little-finger of right hand, singe the neck of tube and innoculate the culture on slant agar with s.c. snake motion.
- Singe the neck of tube and plug, close the tube and singe the loop.

Innoculation on Petri dish into the sectors with s.c. snake motion

- Divide the dish with felt-tip to the sectors from the bottom and sign it.
- Get the culture with loop from the tube (see above).
- Slightly uncover the lid of Petrio dish and innoculate the culture on appropriate sector of agar by pulling of loop on on agar, s.c. snake (fig. 4).
- Cover the dish with lid and singe the loop.

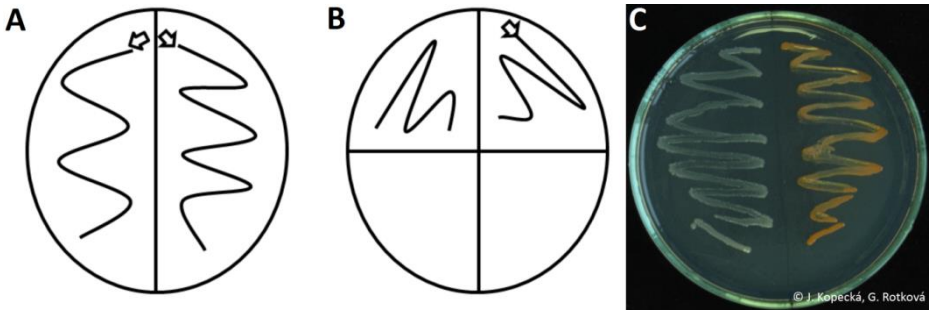


Fig. 4. Design of innoculation on Petri dish s.c. snake-innoculation (A, B) and practical demonstration, *K. rosea* and *P. putida* (C).

Innoculation on Petri dish – streak plate (fig. 5)

- Get the culture with loop from the tube (see above).
- Slightly uncover the lid of the dish and pull the culture on agar by pulling of loop in a few stripes (1).
- Singe the loop, let it cool. Smear the culture by pulling of loop in a few stripes in terminal part of early applied culture (2).
- Singe the loop, let it cool. Smear the culture by pulling of loop in a few stripes in terminal part of early applied culture (3).
- Singe the loop, let it cool. Smear the early applied culture by a fluent move, s.c. snake (4). Single colonies should grow in the place of snake-innoculation (5)

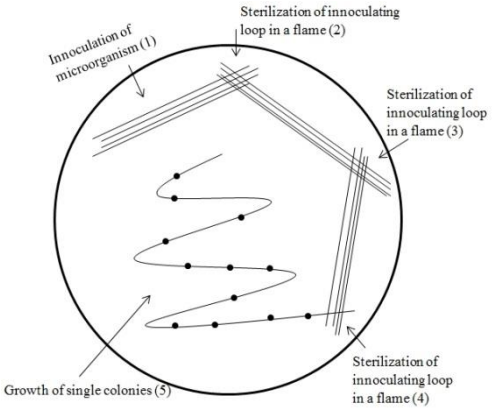


Fig. 5. Streak plate.

Innoculation in to liquid medium

- Get the culture from tube or single colony from Petri dish.
- Take the tube with broth in the left hand. Pull out the plug with little-finger of right hand and singe the neck of tube.
- Smear the culture closely above the the surface of medium and get the culture slowly into the broth.
- Singe the neck of the tube and plug, close the tube and singe the loop.

Innoculation of liquid medium by liquid medium

- Take the sterile pipette in the right hand. Pull out the plug of flask/tube with mixed culture with little-finger. Pippete the target volume of culture.Singe the plug and close the flask/tube.
- Pull out the plug of flask/tube with sterile medium with little-finger of right hand and singe the neck of flask/tube.
- Pipette the culture into the sterile medium, singe the neck of flask/tube and plug. Close the flask/tube and mix gently.

Innoculation from liquid medium on Petri dish

- Take the sterile pipette in the right hand. Pull out the plug of flask/tube with mixed culture with little-finger. Pippete the target volume of culture (usually 100 µl). Singe the plug and close the flask/tube.
- Pipette the target volume in the middle of Petri dishwith agar.
- Smear the culture on surface of agar by sterile stick (L-loop), cover immediately the lid of dish and let it absorb in agar.

Cultivation

- Cultivation is performed at 30 or 37 °C for at least 24-48 hours.

Conclusion

Was the sterility during media preparation in thelast practice prooved? What types of media were used? How was strains innoculated? What is the purpose of streak plate? What temperature is used for strains cultivation? How we choose the right conditions of cultivation? Do the morphology of colonies depends on conditions of cultivation?

Additional information

Miniatlas mikroorganizmů (http://sci.muni.cz/mikrob/Miniatlas/mikr.htm, 17. 7. 2016).

Greenwood D., Slack R. C. B., Peuthere a kol., Lékařská mikrobiologie. GRADA Publishing, 1999, ISBN 80-7169-365-0.

Němec M., Matoulková D., Základy obecné mikrobiologie, Masarykova univerzity, Brno, 2015, ISBN 978-80-210-7923-6.

Sedláček I., Taxonomie prokaryot, Masarykova univerzita, Brno, 2007, ISBN 80-210-4207-9.

Control questions

1. For what we cultivate bacteria on agar in dishes and on slant agar?
2. Write basic phasis of growth curve. Do we observe one single cell or the whole population?
3. Can we evaluate the purity of culture in broth? If not, how you could prove the purity of culture?
4. Why is bacterial culture re-innoculated?
5. What affect morphology of bacterial colony?
6. Does the facultative anaerobe grow in the presence of oxygen? If yes, why?
7. Which groups of microorganism do we divided according to energy source?
8. What are the sources of energy for microorganisms and how do we called the two groups according to these sources?
9. Which way we can select (aids and conditions of cultivation) target group of bacteria?
10. How we can verify „the purity“ of strain by cultivation?
11. What does mean and how is doing the isolation of bacterial strain?

12. What is the principle of streak plate?
13. How is the aseptic work guarateed by re-innoculation of bacteria?
14. What is the reason for no single colonies growth in streak plate (continuous growth of bacteria)?
15. For what we use the selective media?
16. How we divide microorganisms according to relationship to oxygen and to temperature optimum?
17. For what serve innoculation loop and stick?
18. Compare the static and continuous cultivation according to availability of nutrient and accumulation of metabolites.
19. What phase of growth curve is the most appropriate for counting parameters of bacterial growt?
20. What is the difference between obligatory and facultative anaerobe with respect to the tolerance to oxygen?
21. What is the mixed bacterial culture?
22. What is the pure bacterial culture?
23. What is the difference between thermophile and thermotolerant mesophile with respect to the sensitivity to the temperature?

Microorganisms around us

Aim of practice

Demonstate the presence of microorganisms in environment, food or on human body.

Introduction

Microflora is the sum of all microorganisms ocuring in defined space of natural environment, i.e. water reservoir, food, human body. Microflora contains viruses, bacteria and yeasts. Bacterial microflora contains only bacteria. Every environment has its own typical autochthonic microflora, naturally ocuring microorganisms. Natural microflora of *Lactobacillus delbrueckii* and *Streptococcus salivarius* is typical for yoghurts; *Penicillium roquefortii* or *P. camemberti* is typical for cheese (ermines); yeasts *Saccharomyces cerevisiae* or *S. pastorianus* for beer.

Physiological microflora of body is naturally occured on human or animal body or inside the body. It is divided in two groups. Resident microflora is microorganism that colonize human body and do not cause diseases or any damage. Microflora transient contains microorganisms that are present in specific part of macroorganism temporary (oral cavity after food consumation, skin) and that could be pathogenic. Natural microflora defend effectively from colonisation of pathogenic microbes from outside and their reproducing, participates on antigenic stimulation of antibodies formation, especially of immunoglobulins IgA. There is no specific limit for natural microflora – microbes that are natural in one human body can cause disease in another human body.

Microorganisms are used over years due to their ability to reduce the growth of undesirable microorganisms in food (longering of food storage – fermented cabbage, cucumbers, cheese, milk and fruity fermented beverages). Genera *Streptococcus, Lactobacillus, Leuconostoc, Lactococcus, Pediococcus, Enterococcus* (lactic acid bacteria) are widely used in dairy industry for production of butter, yoghurts, cheese and butcher products, acidifying of cream. Yeasts *Saccharomyces cerevisiae* are used for production of top fermented beers (Ale, wheat beer, stout, porter) and wine. Yeasts *S. pastorianus* are used for production of bottom fermenting beers, lagers, due to their ability ferment in lower teperatures.

MRS medium - agar for lactobacilli according to De**M**an, **R**ogosa and **S**harpe with sodium acetate that eliminates growth of other bacteria (except for some lactid acid bacteria, i.e. *Leuconostoc*, *Pediococcus*).

Instruments and microorganisms

Nutrients media on dishes - MPA, MRS, MEA

Sterile cotton-wool swabs, pipette, sticks

Distilled water

Methods

Smears from environment and human body (fig. 6)

- Soak the sterile cotton-wool swab into the sterile distilled water.
- Smear the surface of target object (lips, mobile phone, keys, shoes, skin, …) and immediately smear medium in dish.
- Cultivation is performed at 30 °C for 24-48 hours.

Print from environment or from human body (fig. 6)

- Put the target object (finger, keys, coin) for short time on the agar.
- Cultivation is performed at 30 °C for 24-48 hours.

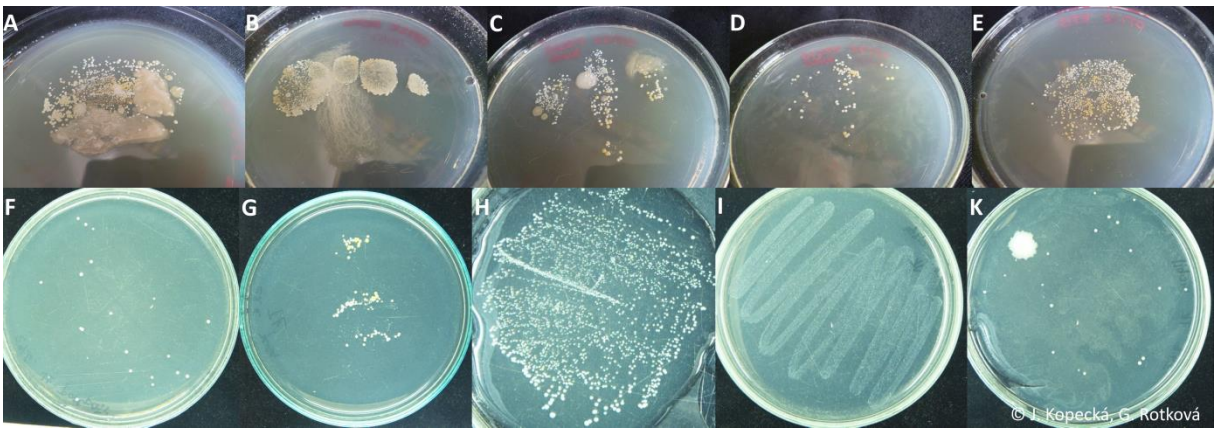


Fig. 6. Smear or print of heel (A), toes (B), fingers before washing (C), fingers after washing (D), lips (E), armpit (F), lips and nose (G), ear (H), mouth (I) and mobile phone (K).

Fallout from air

- Open the dish with sterile MPA on the table (corner of room, at window) for at least 30 minutes.
- Cultivation is performed at 30 °C for 24-48 hours.

Innoculation of beer (nondiluted, nonfiltered)

- Pipette 0,3 ml of nondiluted sample of beer on malt-extract-agar and smear it by sterile stick.
- Cultivation is performed at 30 °C for 24-48 hours.

Innoculation of jogurt, kefir, nonpasteurized milk

- Pipette 0,3 ml of nondiluted sample on MRS agar and smear it by the sterile stick.
- Cultivation is performed at 30 °C for 24-48 hours.

Conclusion

Did the microbes grow on the dishes? What parts of human body showed the most variable species diversity?

Additional information

Klaban V., Ilustrovaný mikrobiologický slovník, Galén, Praha, 2005, ISBN 80-7262-341-9.

Nester E. W., Roberts C. E., Pearsall N. N., Anderson D. G., Nester M. T., Microbiology, A human perspective, WBC/McGraw-Hill, 1998, ISBN 978-0073522593.

Šilhánková L., Mikrobiologie pro potravináře a biotechnology, Academia, 2002, ISBN 80-200-1024-6.

Control questions

1. Which parts of human body are the most colonised and why?
2. Which genera of microorganisms are commonly presented on skin?
3. What is the temporary microflora?
4. Is the microflora of human body dangerous?

Manipulation with microscope

Aim of practice

Manipulation with microscope and with various microscopic techniques.

Introduction

Loupe (magnifying glass) forms direct image magnified 10-15x. Loupe is biconvex or planoconvex len. Loupe serves to observe macroscopical markers of colonies.

Microscope (fig. 7) comprises from mechanical part (base, stand, tale with cross shift), light parts (source of light, condenser, screen) and optical part (objectives, eyepiece). Objective is the set of lens with very short focal distance that forms real conversed image of object.

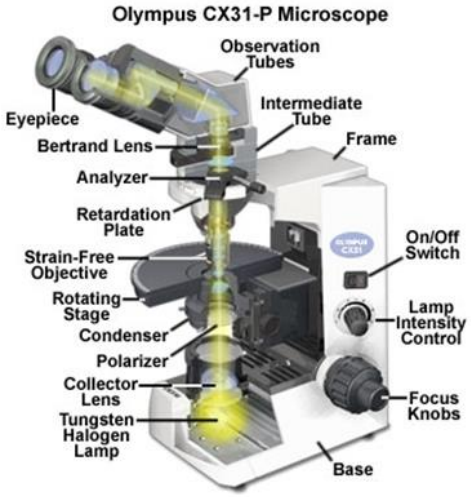


Fig. 7. Parts of the microscope (http://www.olympusmicro.com/primer/techniques/polarized/cx31polconfiguration.html, 12. 7. 2016).

Any waves with shorter wave-length than proportion of object could be used for microscopy. There are 3 types of microscopy - optical, electron and acoustic**.**

Optical (light) microscopy

Dry objective – light ray leaving from preparate in angle α is refracted on interface between cover glass and air and can not participate on image formation anymore.

Immersion objective – light ray passing from glas to immersion environment does not change its direction and could participate on image formation, more rays participate on image formation (fig. 8). Immersion environment is a liquid with the same n as cover glass, mostly

cedar oil (n = 1,52).

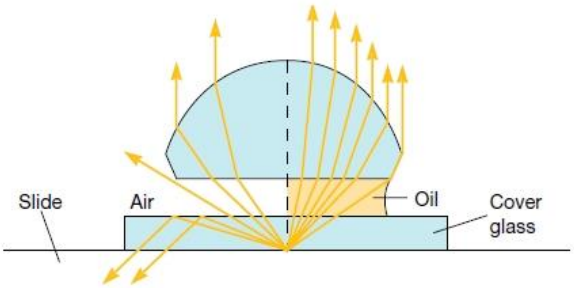


Fig. 8. Passing rays through dry and immersion objective (Prescott, 2013, adjusted).

Formation of image

The base of focused image by lens are light rays spreading from certain point of object to various directions and falling in lens. Rays are converged in one point and compose sharp image of object. Appearance of image depends on distance of object from lens. If the distance of object is two time higher from focal distance, real decreased and inverted image is formed (camera). If object lies between double distance of focal distance and focus, formed image is inverted, real and increased (objective of microscope). If the object lies between focus and len, formed image is increased and unreal (loupe, eyepiece of microsope).

Methods for increase of the imaging contrast are in light microscope:

Eclipse – screen catchs passing rays to objective. Objects are illuminated from side.

Phase contrast (fig. 9) serves for native preparations observing (live nonstained nonfixed cells). Mask is placed on condense with round slot in that light is passing through the object. Phase mask is placed in objective (condenser mask). Semi-permeable metal layer is presented in the place of slot in condenser mask near phase mask that change phase of light about a quarter of wave-length. Nondifracted radiation is passing through from source of part of phase mask that changes light phase. Other waves pass through without any change. This technique converts differences in light phase shift to differences in light intensity that are visible. Shape of cell, movement could be observed. „Dense“ parts of cells with high index of refraction are shining – it is used for spores observing. S.c. „halo effect“ (shiny corona) around cells is characteristic for phase contrast.

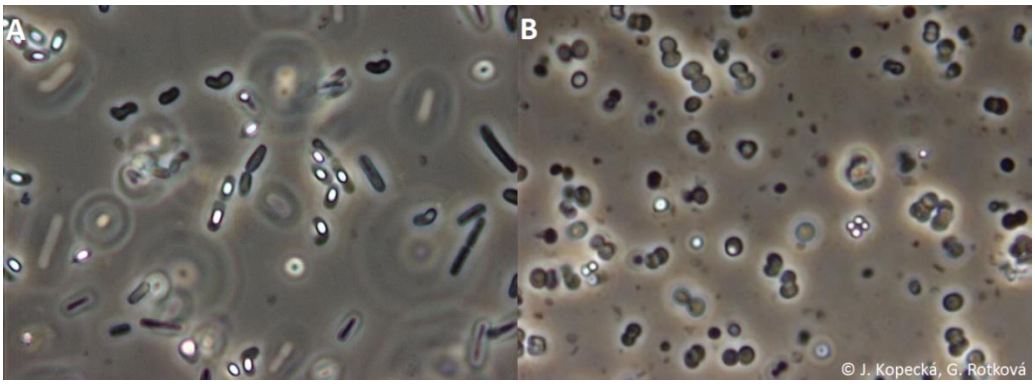


Fig. 9. Phase contrast. *Bacillus cereus* CCM 2010 (A), halo effect around cells and shining spores; *Sporosarcina ureae* CCM 860 (B), only the upper 4 cells producing spores are visible from the sarcina packet.

Nomarski differentioal interference contrast applies with two coherent rays, one is passing through the object, the other is pssing outsite the object. Prism divides originally linear polarised light in two unrighted polarised units. Polarisator arrange waves in various planes. Nomarski plate in condenser is prism that process polarised light, two rays go simultaneously side-by-side on preparation. 3D image could be seen in analyser in dependence on various index of refraction of various cell parts. Plastic image of cell surface is formed by highlighting of small differences (fig. 10).

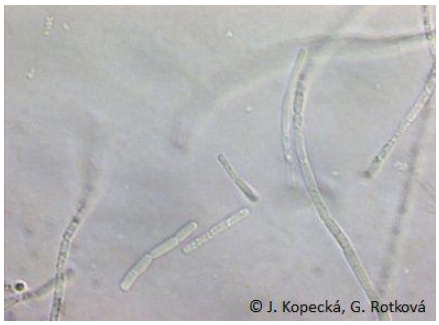


Fig. 10. Nomarski contrast, *Bacillus cereus.*

Methods

- Turn on the source of light and get the preparate on the table.
- Regulator of light and condensor is not needed to regulate.
- Find the object by makro-screw and focus by micro-screw.
- Increase the magnification gradually by changing the objectives on revolver, objective is not allowed to collide with the cover glass!

- Objectives on revolver are usually parfocal (focused on the same distance). I tis not necessary re-focus the object after objective changeing, i tis necessary only focus by micro-screw.
- Revolver-changer serves for choice of suitable objectives.
- Objectives designated „Ph“ serves for observing of preparates by phase contrast.
- It is always necessary to use the immersion oil for objectives with black and white stripe (objective 100x)! The immersion oil is not used for objectives 10x, 20x and 40x!
- It is necessary clean the objective after usage of immersion oil!

Usage of immersion oil

- Focus the object by the highest magnification nonimmersion objective (mostly 40x).
- Turn the revolver in position between nonimmersion and immersion objective. Put the drop of immersion oil on the preparate.
- Turn the revolver on **immersion objective**, the objective must be **dipped into oil**. Focus by micro-screw.
- If the object is not seen, it is necessary draw the objective close to the preparate, but not to collide with it. Watch the objective and table with preparate from the side.
- Subsequently, move the objective slowly from preparate and observe until the object is visible in eyepieces. If the object is still not seen, we have to repeat the focusing.

Conclusion

Do you succeed in focusing? Was there any difference among observing preparates in brief fild, phase and Nomarski contrast?

Additional information

Matis D., Mikroskopická technika. ISBN: 80-968522-0-5, 2001.

Plášek J., Nové metody optické mikroskopie. Pokroky matematiky, fyziky a astronomie, 1996, 41: 1-24.

Harley J., Laboratory exercises in mikrobiology, 2013, ISBN-10: 0077510550.

Hrazdíra I., Mornstein V., Lékařská biofyzika a přístrojová technika, Neptun, Brno, 2004, ISBN-10: 80-902896-1-4.

Virtuální mikroskopování (http://olympus.magnet.fsu.edu/primer/virtual/virtual.html, 18. 2. 2016).

Vlnová optika (http://www.sweb.cz/radek.jandora/f11.htm, 28. 7. 2016)
Davidson M., W., Abramowitz M., Optical microscopy (https://cw.fel.cvut.cz/, 28. 7. 2016)
wiki/_media/courses/a6m33zsl/davidson-abramowitz-optical_microscopy.pdf (18. 2. 2016)

Control questions

1. Why we should not use after usage immersion oil non-immersion objective?
2. For what serves immersion oil?
3. What is basis of the phase contrast?

Macroscopic and microscopic observation of microorganisms, Gram staining

Aim of practice

Evaluation of microbial cultures morphology - macroscopically. Gram staining. Observing and evaluation of microscopic preparations.

Introduction

Morphology and cytology help to identify microbes, to control contamination of cultures and to assess physiological state of culture. Macroskopic and microscopic characteristics are evaluated always parallel.

Macroscopic characteristics

acroscopic characteristics could not be evaluated in **liquid media**. Only the character of growth could be evaluated in liquid media (fig. 11) by presence of sediment (optional aerobic culturesy), diffusion turbidity (aerobic cultures have turbidity in the whole medium), upper layer on surface (poor wetting force of cells or mycelium, surface tense, in most cultures forming rough or wrinkled colonies), rough flocs (aerobic cultures), membrane at surface (surface formation of sediment forming yeast, produced ethanol is utilised after sugar depletion) and pigmentation.

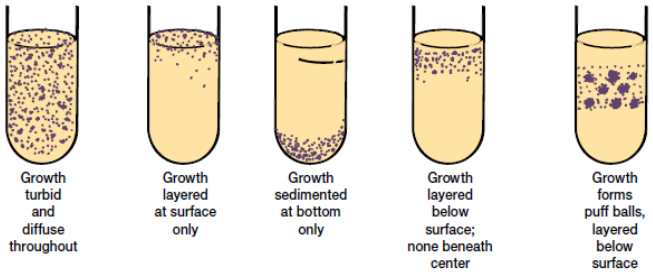


Fig. 11. Growth of microorganisms in broth (Prescott, 2013, adjusted).

Macroscopic evaluation lies in colonies description. Appearance of colonies is influenced by type of nutrient media, age of culture and type of cultivation.

Single colonies can not be evaluated on **slant agar**. Speed of growth, shape of smear (straigh, full, pointed, rhizoid), profile of smear (flat, bulge), surface of smear (shiny, rough, dry), conzistence and pigments could be evaluated. Requirements of microbes to oxygen could be

determinated by stab in slant agar: aerobic microbes grow in upper part of stab, anaerobic at bottom, optional anaerobic over the whole length of stab.

Colony is the clone of cells growing from one single cell. **Single colonies** (fig. 12) could be observed and evaluated after right prepared streak plate on **Petri dishes**: size (diameter; mm), shape (regular, round, oval, irregular, lobate, filamentous, rhizoid), profile (elevated, flat, umbiliform, dish-shaped), margin (regular, filiform, lobate, circular), surface (smooth, bright- S – phase, shimmer, rough - R- phase), transparency (transparent, translucent, nontransparent), colour (colourless, pigmentation). Another characteristics are smell, odour (jasmine, butter, fruity), mycelium formation, medium changes (colour, hemolysis, precipitation), consistence (viscous, smeary, friable, growing into agar).

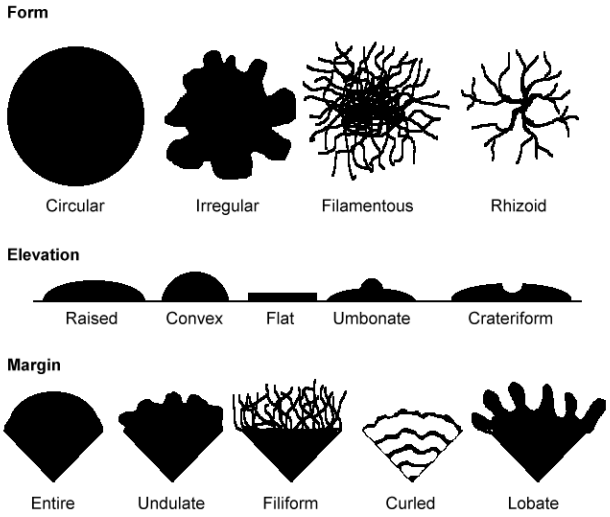


Fig. 12. Shapes of bacterial colonies (http://www.sciencebuddies.org/science-fair-projects/project_ideas/MicroBio_Interpreting_Plates.shtml, 12. 7. 2016).

Inequality of colony growth could be caused by age of culture. Mucoid (M) character of growth is manifested as wet, mucous and very lustrous colonies formed mostly by cells with capsules (i.e. *Azotobacter*, *Leuconostoc*). Smooth (S) colonies have straight margin with or without lustre in noncapsulated cells. Rough (R) colonies have dry, irregular margins, are various wrinkled. Cells forming chains, mycelia or pseudomycelia (i.e. *Bacillus, Trichosporon,* yeast *Pichia, Hansenula, Candida*) grow in rough colonies. Changes in type of colony M – S – R are caused by mutations.

Microscopic characteristics

Shape, size and clustering of cells, presence of special formations on cell, type of

reproduction visible in preparation are evaluated in microscopic preparations. Preparations could be observed in native preparation in phase contrast or the morphology of cell and its structures could be highlighted by fixation and staining (Gram, Ziehl-Nielsen staining). Cover glass is always used for native preparation (observing of suspension). Glass cultures (cover glass overgrowed by cultivation) are used for moulds, yeasts or actinomycetes observation.

Cells have various shapes (fig. 13): cocci (spherical, flattened, diplococci, streptococci, tetrads, sarcina, staphylococci), rods (straight, curved, branched, palisades, pleomorphic), cocobacilli, cells with buds or stalk, spirilli, stars, mycelia. Size of target bacterial cells is shown in Tab. 1.

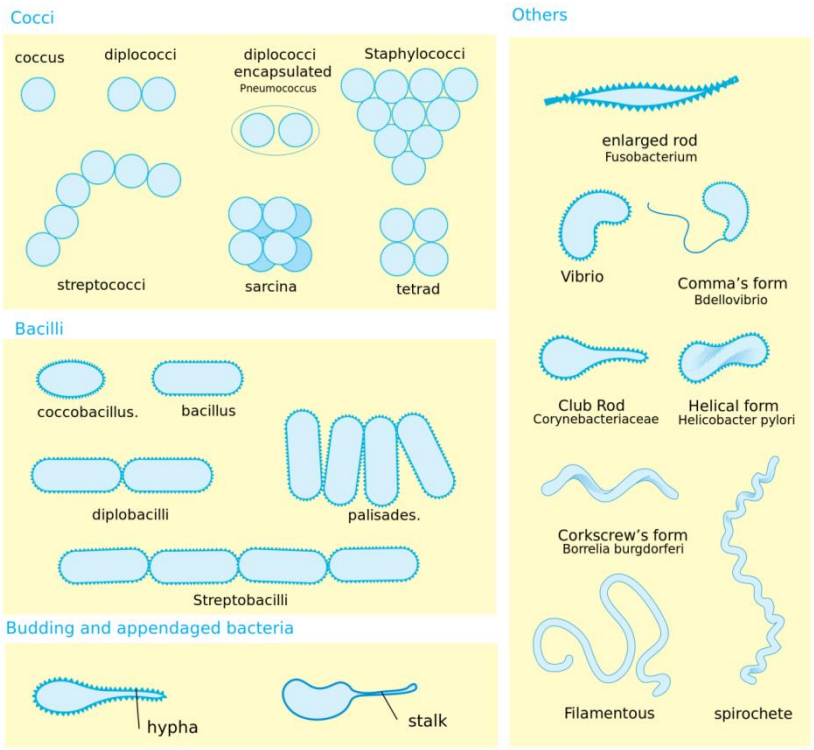


Fig. 13. Shapes of bacterial cells (https://en.wikipedia.org/wiki/Bacterial_cellular_morphologies, 24. 2. 2016).

Tab. 1. Size of bacterial cells.

Microorganism	**Size (µm)**
Chlamydia	0,3 * 0,3
Bdellovibrio	0,8 * 0,3
Rickettsia	1 * 0,3
Staphylococcus aureus	0,8-1 * 0,8-1
Escherichia coli	2-3 * 0,4-0,6

Bacillus subtilis	1,8-4,8 * 0,9-1,1
Streptomyces	filament * 0,7-1,6
Chromatium	25 * 10
Spirochety	500

Native preparation

Native preparation is never fixed. Preparation is not stained, serves to evaluate real shape and structure of undamaged cells. It is used for growth, reproduction and movement of bacteria observation, for study of cell formations that are stained with difficulties, i.e. spores. Brief field, Nomarski or phase contrast are used for structure observation.

Fixation of preparation

Nature of fixation is to precipitate cell coloids, especially proteins. Cells adhere better to microscopic slide, are not washed by dye or solvent application and absorb dye better after fixation. Preparation is fixed when the smear of cells is perfectly dry to avoid cell boiling. Fixation is performed by pulling the microscopic slide through the flame. The smear of cells have to be on upper side of slide. If cells were cultivated im sugar liquid medium, it is necessary to separate them by centrifugation and to wash them by water of buffer. Yeast and mould cells are bigger than bacterial cells, thermal fixation could modify their shape. They are mostly fixate by chemicals. Fixation and staining of cell slightly deform cells, but their characteristic shape remains. Nonfixed, negative stained (staining of background of cells) preparation is used to measure accurate size of cells.

Stained preparations

Stained preparations serve to determinate type of cell wall, shape of cells and their clustering, presence and location of spores, presence of capsule and internal cell structures (inclusions) and vitality of cells. Morphology of cell and characteristic clustering could be observed after simple staining of cell wall (i.e. by crystal violet) without differentiation of grampositive or gramnegative type. Vital test shows ration of live and dead cells in nonfixed preparation. Vital staining stain dead cells that absorb dye (i.e. diluted Löffler blue) and do not secrete it outside by efflux systems. Cellt structures are differentiate by differentiating staining, as internal as outside formation (spores, capsules, cell walls), chemical compounds (volutin, glycogen, starch). Diagnostic staining hels to identify bacteria (i.e. Gram, acid-fast staining by carbolfuchsin, staining according to Giemsa). Cells are not fixed and stained at negative

staining, only the background is stained (i.e. by nigrosin). It is used to measure accurate cell size of undamaged cells.

Preparation is fixed every time before staining except for negative staining and vital test. Diluted water solutions of organic dyes, commonly salt, are used for stining. Basic dyes have colour cationt, acid dyes aniont. Basic dyes (i.e. crystal violet, methylene blue, safranin, basic fuchsine, malachite green) are mostly used for bacteria staining. Staining could be highlighted by chemical treatment of cells (i.e. by phenol, tannin). Chemicals have higher afinity to cell and to dye than the single cell afinity to dye.

Gram staining

Gram staining is one of the most important diagnostical methods for identification of bacteria. It differentiates the group of grampositive (stained blue-violet) nda gramnegative cells (stained red or pink) ans show some physiological and chemical characteristics of cell. The nature of various behaviour during Gram staining was not yet satisfactory described, however, he diffences in cell wall composition of both groups of bacteria play key role. Fixed preparation is stained by crystal violet and subsequently treatd in KI solution. Complex dye-iodine-cell wall is formed. Difference arises during washing of preparation by organic solvent (acetone or alcohol). Solvent dissolves the outer lipopolysaccharides layer, complex of crystal violet-iodine is washed out through thin peptidoglycan layer and cells are colourless, in the case of gramnegative culture. While grampositive bacteria maintain the dye in cell. Another contrast dye is used to highlight the difference (i.e. basic safranin, carbolfuchsin) to colour gramnegative cells in red, pink. Grampositive cells have already dye (crystal vilet) binded in cell wall and remain coloured in blue-violet. Mistakes during Gram staining are: too dense smear of cells; boiling of cells during fixation; too long washing of cell by alcohol.

Gram staining is affected by physiological state of cells, age of culture and media composition. The most suitable are 24-hours old cells. Cells could loose their grampositivity i.e. by mechanical damage, UV radiation, acid, base or solvent treatment. Microorganisms, partly stained as grampositive and gramnegative at the same time in pure culture, are called gramlabile/gramvariable. Some bacterial genera can not be stained by Gram staining, there are genera without cell wall (mycoplasma), spiral bacteria and strong acid-fast genera (mycobacteria); i.e. *Borrelia burgdorferi, B. recurrentis, Bartonella henselae, Chlamydia trachomatis, C. pneumoniae, Chlamydophila psittaci, Coxiella burnetii, Ehrlichia chaffeensis, Anaplasma phagocytophilum, Legionella* sp., *Leptospira* sp., *Mycobacterium bovis, M.*

tuberculosis, M. avium, M. intracellulare, M. kansasii, M. leprae, M. marinum, Rickettsia rickettsii, Orientia tsutsugamushi, Treponema pallidum.

Cell wall of bacteria

Gramnegative type (fig. 14B) of cell wall is composed from outer lipopolysaccharides membrane and inner relative thin peptidoglycan (circa 5-10 % of cell wall) layer containing muramic acid. Lipoproteins connect peptidoglycan and outer membrane. Lipopolysaccharides are composed from lipid A, core (base) polysaccharide and O-antigen (O-chain). Outer membrane serves as defend barrier against to environment, permeation of compound or slow down the permeation (bile salts, antibiotics, toxins.).

Cell wall of grampositive type (fig. 14A) lacks outer membrane and peptidoglycan layer is relative thick. Some strains could contain as part of cell wall teichoic, lipoteichoic acid, neutral polysaccharides or mycolic acids.

Special bacterial group are bacteria lacking cell wall, s.c. mycoplasma that are not capable to synthetise precursors of peptidoglycans. Cells are coated only in plasma membrane.

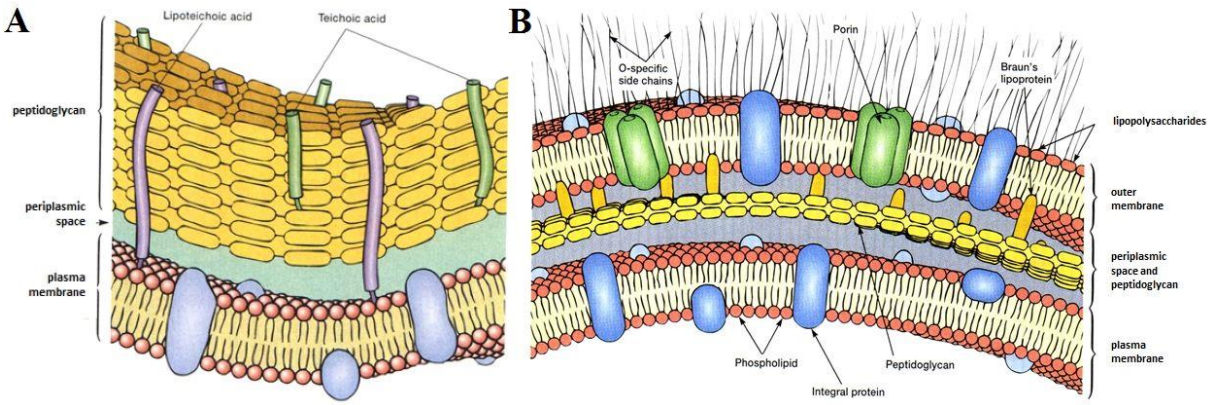


Fig. 14. Cell wall of grampositive (A) and gramnegative (B) type (Prescott et al., 1996, adjusted).

Instruments and microorganisms

Microscopic slide, cover glass, innoculation loops, loupes

Dyes for Gram staining (crystal violet, Lugol solution, safranin)

Distilled water

Escherichia coli CCM 3954

Pseudomonas putida, P. fluorescens

Serratia marcescens CCM 303

Kocuria rosea CCM 839

Micrococcus luteus CCM 169

Bacillus cereus CCM 2010

Bacillus subtilis CCM 2216

Staphylococcus aureus SA 812

Saccharomyces cerevisiae

Methods

Everybody works independently in this practice.

Sign preparations on glass or on paper sticked on glass.

Everybody evaluates and stains:

- Morphology of colonies and growth on agars and in broth
- Gram staining – pure and mixed culture
- Observe microscopically all cultures in practice, evaluate the shape of cells, clustering of cells and the type of cell wall
- Native preparation

Native preparstion - aseptic work during putting the cells on glass

- Pull out the cleaned microscopic slide from alcohol and pull it through the flame.
- Put the drop of sterile distilled water in the middle of microscopic slide.
- Get a little amount of culture into the drop of water by singed, cooled loop and mix it. Use only the small amout of culture to have adequately dense preparation.
- The drop is not smeared, but is covered by cover glass without the bubbles (not cover from above, but at first put the one edge into the preparation, do not push).
- Remove the redundant liquid by filter paper.
- Observe the cell from liquid medium direct in medium without the dilution in drop of water.
- Choose the phase contrast objective (Ph) for native preparation.
- Observe the preparation until 5 minutes (quick drying).

Gram staining

- Put out the cleaned microscopic slide from alcohol and pull it through the flame.

- Drop sterile distilled water in the middle of microscopic slide.
- Get a little amount of culture into the drop of water by singed, cooled loop and mix it.
- Smear the suspension onto the glass, let it dry and fix it by the flame (pull the glass through the flame for three times).
- Put the preparation in the solution of crystal violet (30 seconds), wash the dye by water.
- Put the preparation in the Lugol solution (30 seconds), wash the dye by water.
- Wash the preparation by ethanol (or acetone), max. for 15-20 seconds.
- Was the preparation by water.
- Put the preparation in the safranin for 1 minute (only gramnegative cells are stained by safranin, because crystal violet was decoloured; it is necessary to stain every preparation by safranin – we no not know how type of cells is in the preparation).
- Dry preparation by filter paper and observe at magnification 1000x (immersion objective) in brief field (BF objective).

Conclusion

Did you succeed in bacteria staining and observing? If not, why?

On fig. 15, 16 and 17 you can see streak plates and microscopic preparations (Gram staining) of cultures used in practices.

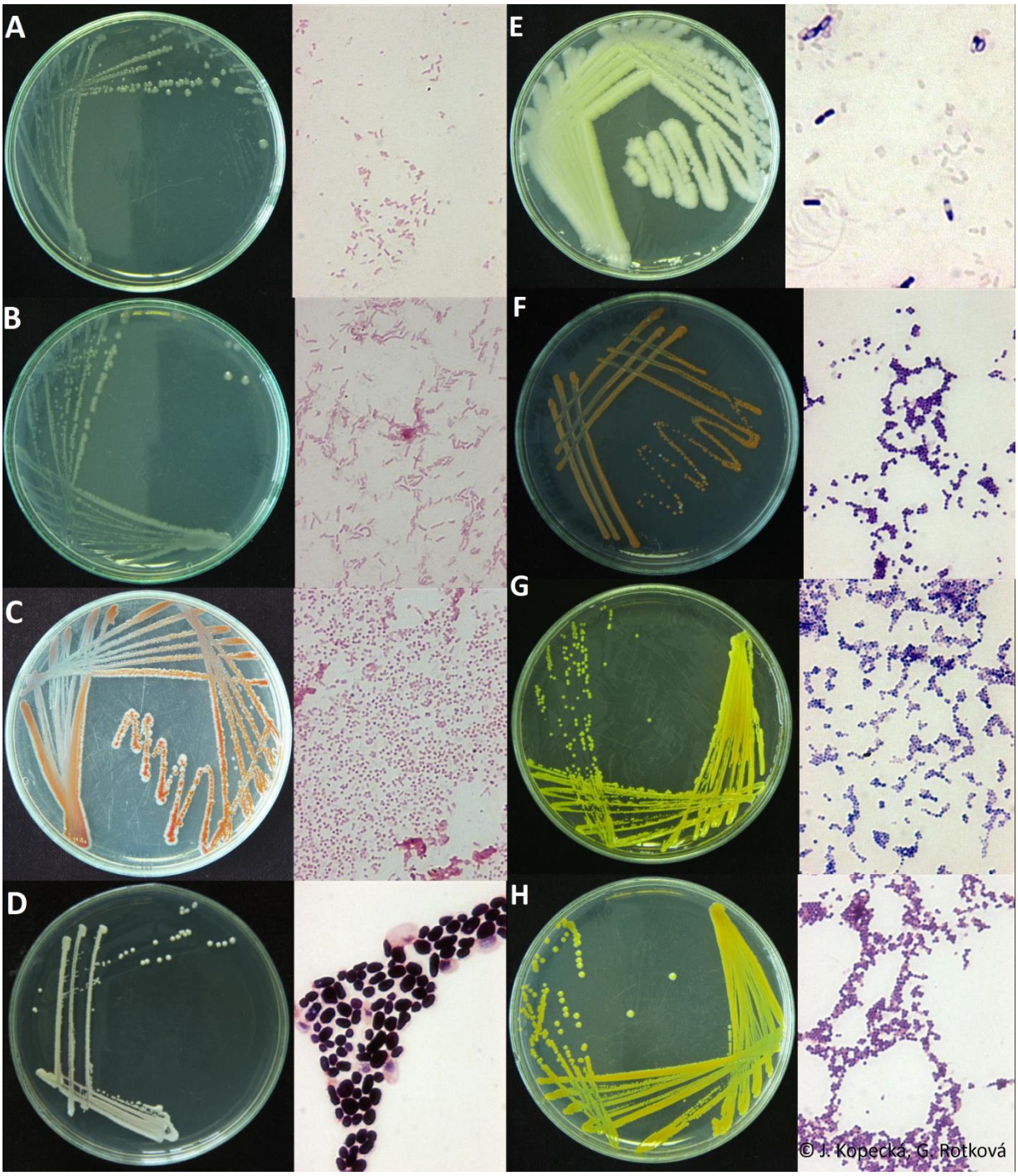


Fig. 15. Pure cultures of bacteria on agar and stained according to Gram. *Escherichia coli* CCM 3954 (A), *Pseudomonas putida* (B), *Serratia marcescens* CCM 303 (C), *Saccharomyces cerevisiae* (D), *Bacillus cereus* CCM 2010 (E), *Kocuria rosea* CCM 839 (F), *Micrococcus luteus* CCM 169 (G), *Staphylococcus aureus* SA 812 (H).

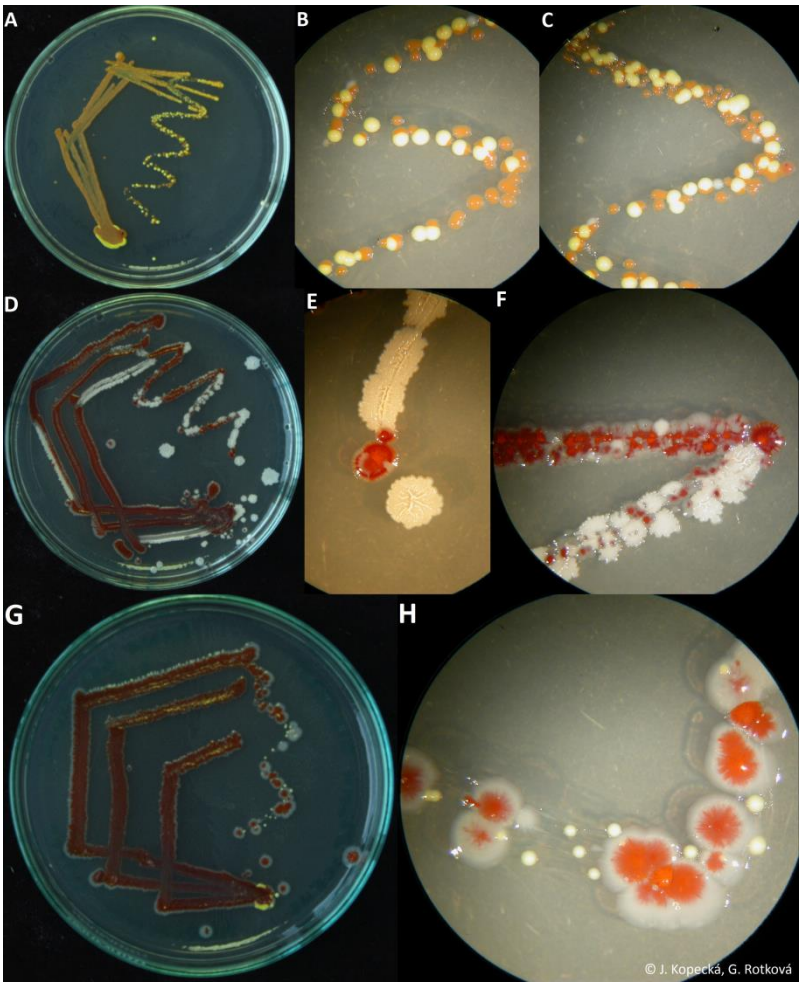


Fig. 16. Mixed cultures of bacteria. *M. luteus* CCM 169 and *K. rosea* CCM 839 (A, B, C), *S. marcescens* CCM 303 and *B. subtilis* CCM 2216 (D, E, F), *S. marcescens* CCM 303 and *M. luteus* CCM 169 (G, H).

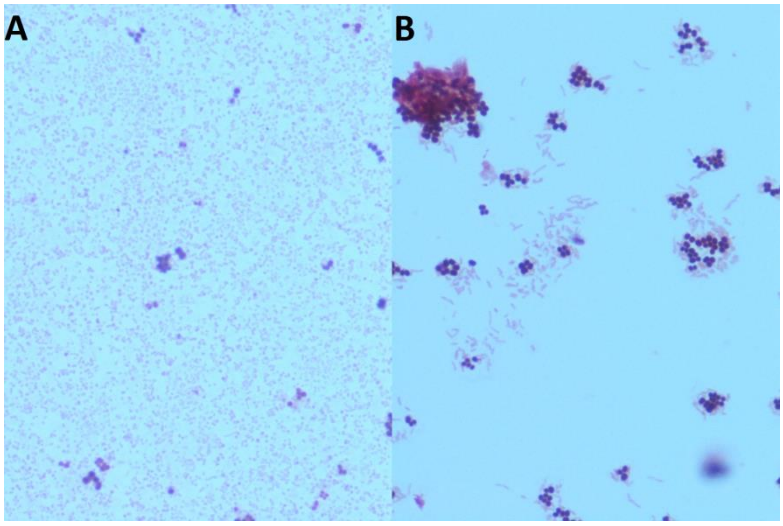


Fig. 17. Mixed cultures stained according to Gram. *S. marcescens* CCM 303 and *K. rosea* CCM 839 (A), *M. luteus* CCM 169 and *P. putida* (B).

Additional information

Miniatlas mikroorganizmů (http://sci.muni.cz/mikrob/Miniatlas/mikr.htm, 23.2.2016).

Prescott L., M., Harley J. P., Klein D. A., Microbiology, WCB, Dubuque, 1996, ISBN 0-697-29390-4.

Rosypal S., Obecná bakteriologie, SPN Praha, 1981.

Šilhánková L., Demnerová K., Návody pro laboratoře z mikrobiologie, VŠCHT, Praha, 1993.

Atlas mykologie (http://www.mycology.adelaide.edu.au/, 23. 2. 2016)

Centers for Disease Control and Prevention (http://www.cdc.gov/az/a.html, 23. 2. 2016).

Control questions

1. Which dyes are used during the Gram staining?
2. What we can dye in preparation (cell)?
3. Which morphological characteristics of single microbial colonies are described?
4. Are every bacteria stained/dyed according to Gram staining? If not, write some examples.
5. On what depends morphology of bacterial colony?
6. Why is necessary fo fix the preparation?
7. What is the bacterial species? What is the type culture of bacterial species, where is deposited?
8. Write at least 2 examples of bacterial genus forming cocci and rods.
9. Which structure of cell is stained by Gram staining?
10. Which objective provides higher magnification – immersion or dried objective? For what serves immersion oil?
11. How are the yeasts stained according to Grama staining; which type of cell wal have yeasts?
12. How is *Escherichia coli* stained according to Grama staining?
13. What is peptidoglycan?
14. What are the main differences between preparation for Gram staining and native preparation in term of procedure and aim?
15. For what serve various types of cell dying?

Bacteriophage

Aim of practice

Determinate the titre of phage lysate by two-layer-agar method.

Introduction

Bacteriophages (also phages) are viruses infesting bacterial cells. Size of bacteriophages ranges from 20 to 200 nm, size of genome from 2 to 200 kb, they are smaller than bacteria. Number of phages in environment is 10 times higher that number of bacterial cells. Phages couse mortality of bacteria in 20 – 50 %, help to compounds circulation, increase the amount of organic compounds in environment. Phages have not the same genetic information together with animal and human viruses. Evolution of phages takes place together with evolution of bacteria.

Phage nucleid acid (single- or double-stranded, circular or linear, DNA or RNA) is deposited in protein capsid with mostly shape of icosahedron that is connected with tail by collar. Tail fibers are attached on basal plate, fibers serve for attachement on surface receptors of bacterial host (fig. 18).

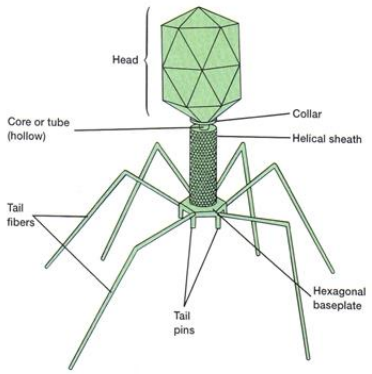


Fig. 18. Structure of T4 bacteriophage (Prescott et al., 1996, adjusted).

Range of bacterial host could be very specific or broad. Some phages are so much specific and multiply in only specific strains of definite bacterial species. Phages with broad spectrum of hosts are polyvalent. Chimeric phage originats by homologous recombination between two phages, has characteristic of both ancestral phages.

Two groups are distinguished according to phage life cycle:

Virulent phages with lytic life cycle (fig. 19A) cause lysis of host bacterial strain: 1. Adsorption of virion on surface of sensitive cell, penetration of phage nucleid acid in cell cytoplasm, 2. Replication of phage DNA using RNA polymerases of host, synthesis of early phage mRNA a proteins that control synthesis of additional viruses, synthesis of late proteins of phage, 3. Completation of new viral particles (virions), 4. Releasing of virions in environment caused by endolysin synthesis and cell lysis. Released virions could infect another sensitive cells. If lytic infection takes place in liquid suspension with host cells, medium is clariffied by infection – s.c. phage lysate (cultivation environment containing active viral particles, rest of cell membranes, cell content). If lysis takes place on solid medium with cells, plaques originate.

Temperated phages with lysogenic cycle (fig. 19B) are incorporated in host genomy by recombination, phage nucleid acid is replicated and transferred to offspring together with bacterial genome. This phage is called prophage, could be released from DNA of bacterial cell after some factor treatment (UV radiation, mutagens or stress factors). Together with phage genetic information, part of host genome could be splitted by wrong reading frame shift and become of part of new phage particle. This process is called transduction and i.e. in species *Staphylococcus aureus* it is the most frequent methods of horizontal gene transfer that could bring some advantageous genes to virus. Transduction is advantageous also for bacteria because of serotype-converting phages.

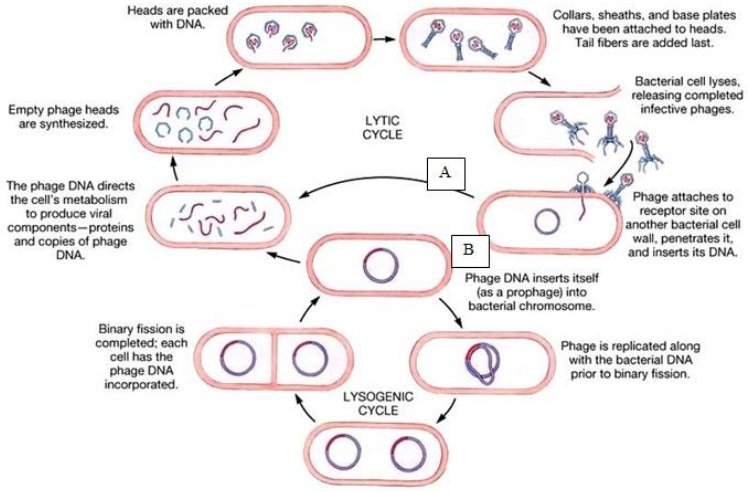


Fig. 19. Life cycle of phage (http://vle.du.ac.in/mod/book/print.php?id=9808, 25. 2. 2016, adjusted).

Infection of bacteria by phage is not always succesful. Some bacteria cover their receptores for phage binding by protein A or activate their restriction modification system and

endonucleases cleve phage nucleid acid. That are the main problems of phage therapy. Solution is the isolation of phage mutants that are capable to overcome the defend mechanisms of host and lyse originally insensitive cells.

Phage therapy is usage of phages for bacterial infection treatment. Bacteriophages capable of pathogenic bacteria lysis are important due to resistant bacteria to reserve antibiotics (i.e. methicillin).

Bacteriophages were used to treatment and prevention of infection in past. Formerly were used preparates of whole phage particles, today could be used isolated and well characterised purificated phage components with antimicrobial characteristics. Phage therapy is developed especially for infectin caused by genera *Staphylococcus, Streptococcus, Pseudomonas, Proteus, Klebsiella, Shigella, Salmonella, Escherichia, Enterococcus* and *Listeria.* Advantage of therapy is high specifity to target strains of host, phages are not dangerous for natural microflora of patients. Phage specifity requires very exact diagnosis of pathogen and assessment of its sensitivity to target phage. Polyvalent phages or mixture of phages could be employed to bacterial lysis. Bacteriophages are capable to reproduce in place of infection, mostly one sigle preparate is sufficient compared with antibiotics.

Phage therapy is used in medicine, but also in food treatment, in phytotherapyplant breeding and plant production against bacterial infection of plants.

Indirect methods based on plaques ormation are used to determinate number of phage particles. Plaques are clariffied zones on solid medium overgrowed by host cells. Plaques originate on place where was one single active phage particle in time of innoculation that reproduces and infects another sensitive cells. The base principle of method is to have higher amount of host cells than phage particles.

Instruments and microorganisms

Staphylococcus aureus SA 812, staphylophage 812

Meat-peptone-broth (MPB)

Meat-peptone-agar (MPA) – 2 % a 0,7 %

sterile tris HCl buffer (pH 7,2)

sterile 0,22 % $CaCl_2$

sterile Petri dishes, pipettes, tubes, water bath, thermostat

Methods

Preparation of phage lysate

- Innoculate 2 ml of 24-hours inoculum of *S. aureus* SA 812 into 100 ml of MPB in aeration flask. Cultivate the mixture another 4 hours at intensive aeration at 30 °C.
- Add aseptically 10 ml of sterile 0,22% $CaCl_2$ and 5 ml of stock solution of phage lysate 812 and continue in cultivation.
- Transfer the aeration flask in dark at room temperature after 60 minuts.
- The medium is getting clear after 12 – 24 hours, however small number of insensitive cells remains in solution.
- Sterilize the lysate by adding of chloroform (5 - 10 drops/10 ml of lysate), allow the chloroform work for 1 - 2 hours. Transfer the lysate by sterile pipette into sterile flask.
- Number of active phage particles is not significantly changed at 4 °C over 1 - 2 months.

Preparation of host cells

- Innoculate stock solution of *S. aureus* SA 812 into the 20 ml of sterile MPB (in 100 ml flask) and cultivate for 24 hours at 30 °C.

Assessment of number of virions

- Dilute the phage lysate 812 in sterile tris-HCl buffer: pipette 0,1 ml of lysate or previous dilution into 0,9 ml of buffer, for each transfer use the new sterile tip, mix the solution properly!
- Prepare sterile tubes containing 3 ml of 0,7% MPA, boiled and tempered at 45 °C.
- Add aseptically 2 ml of 0,22% $CaCl_2$ to 20 ml of host cells and pipette 0,3 ml of inoculum into each tube.
- Pipette 0,1 ml of target lysate dilutions on dishes with 2% MPA, pour immediately the dish by one tube (MPA, $CaCl_2$, host cells), mix carefully by circular movement and let it solidify.
- Cultivate at 30 °C for 12 - 24 hours.

Evaluation

Count the number of plaques on dishes (fig. 20) with appropriate dilution (circa 20-200 plaques/dish). Dish with less than 10 plaques is not suitable for evaluation. The result is in

PFU/ml units (plaques forming units, number of phage particles capable to form plagues in 1 ml, not the absoluten umber of virions).

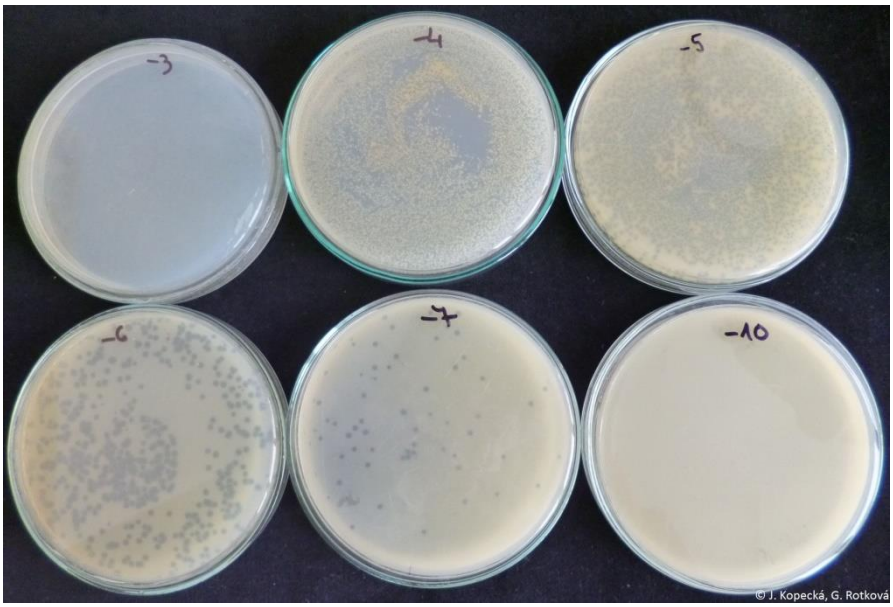


Fig. 20. Plaques growing after innoculation - set of dilution of phage lysate (dilution from 10^{-3} to 10^{-10}).

Every cell is lysed in dilution 10^{-3}, in contrast no lyse is observed in dilution 10^{-10}. The appropriate dilution is $10^{-7}$for the counting of plaques.

Example of calculation: The total number of 122, 132, 139 plaques was formed on 3 dishes (0,1 ml of sample, dilution 10^{-5}/dish), arithmetic average is 131 plaques**.**

Titre of lysate is = 131 * 10^5 = **1,31 * 10^7** in **0,1 ml**, also **1,31 * 10^8 PFU/ml**.

Additional information

Eyer L., Pantůček, R., Růžičková V., Doškař J., Nové perspektivy fágové terapie, přehledový článek v Klinická mikrobiologie a infekční lékařství, 2007.

Jandová B., Kotoučková L., Praktikum z mikrobiologie, ISBN 80-210-1374-5 (http://www.sci.muni.cz/mik/wp-content/uploads/mikrobiologiecv.pdf, 17. 7. 2016).

Otradovcová L., Izolace a charakterizace mutant polyvalentního stafylokokového bakteriofága účinných proti methicilin-rezistentním kmenům *Staphylococcus aureus*, Diploma thesis, MU, Brno, 2012.

Control questions

1. Write some differences between plant and animal viruses.

2. What is the significance of viruses in term of transfer of genetic information in environment?
3. What is the advantage of viruses in therapy?
4. What is necessary for bacteriophage cultivation?
5. For what is $CaCl_2$ during the phage cultivation?
6. Explain the difference between lytic and lysogennic cycle.
7. On what are the viruses cultivated in general?
8. What is the plaque?
9. What is the result of infection by virulent phage in broth?
10. What is the result of infection by virulent phage on solid agar?

Bacteriophage – transduction

Aim of practice

Verify the right process of transduction by transferring the antibiotic resistance from donor to the recipient bacterial cells.

Introduction

Transduction is horizontal genetic material transfer due to virus from donor cell to recipient cell. Any fragment of chromosome could be transferred from donor bacterial cell to recipient cell by bacterial viruses (bacteriophages, phages). Transferred fragment recombinates with homological part of recipient cell, integrates in cell genome that is showed in its phenotype. Mechanism of transfer is not well described, it is probably mistake at DNA packaging in phage head during phage particles compilation in infected cell. DNA of donor cell, part of chromosome or plasmid could be packed in phage head instead of replicated phage DNA and could be transferred to recipient cell.

Over 250 of staphylococci bacteriophages is describes in literature today. Capability of transduction has only some of them (serologic group B and sibling group F). Prototype of transduction phage is phage φ11 from serologic group B that is capable to pack up to 45 kb of chromosomal or plasmid DNA (size of its genome) in its head. Induction of phage is caused by UV radiation or mitomycin C from chromosome of strain NCTC 8325, where is integrated in from of prophage. Another bacteriophages with high transduction efectivity are phages 52A, 53 and 80.

Transduction is used to target transfer of mobile genetic elements to strains *Staphylococcus aureus*, to prepare modified variants of strains to study of horizontal gene transfer and to identify transferred genes.

Instruments and microorganisms

Donor strain *Staphylococcus aureus* Jevons B (contain 2 plasmids, resistance to cadmium and tetracycline)

Recipient strain *Staphylococcus aureus* RN4220

Transduction phage JB

MPA, MPB

$CaCl_2$ solution, sodium citrate

tetracycline
centrifuge

Methods

Preparation of transduction phage lysate

- Innoculate the donor strain (20 µl of stock solution into 20 ml MPB) and incubate at 37 °C for 18 hours.
- Transfer 2 ml of 18-hours culture into 50 ml of fresh MPB in aeration flask and incubate at 37 °C for 2 hours at intensive aeration.
- Add 6 ml of phage lysate, solution of $CaCl_2$ (final concentration 2 mM) and incubate at the same conditions, at 37 °C for 2 hours at intensive aeration (stock solution of $CaCl_2$, concentration 0,02 M, for final concentration add 1/10 of volume).
- Store the lysate overnight at 4 °C (complete the lysis, in the case of completed lysis of bacterial culture immediately after incubation, this step could skipped).
- Remove the rest of donor strain cells by centrifugation (5000 rpm / 30 minutes) and filter through 0,45 µm bacteriological filter.
- Assess the titre of phage particles in lysate by method of 2-layer agar (for calculation of frequency of transduction).

Transduction of plasmids

- Innoculate the recipient strain (20 µl of stock solution into do 20 ml MPB) and incubate at 37 °C for 18 hours.
- Assess the titre of recipient strain (for calculation of frequency of transduction).
- Add solution of $CaCl_2$ to the culture of recipient strain to final concentration 2 mM (stock solution of $CaCl_2$ has concentration 0,02 M, for final concentration add 1/10 of volume).
- Mix 1 ml of recipient cells with 1 ml of transduction phage lysate, the value of infection multiplicity will be max. 1 (ratio of number of phage particles to number of cells).
- Incubate the transduction mixture at 37 °C for 25 minutes constantly shaking.
- Add solution of sodium citrate to transduction mixture to final concentration 15 mM, centrifuge (3000 rpm / 10 minutes / 4–8 °C) and resuspend the pellet in solution of 17 mM sodium citrate. Volum efor resuspendation is depend on number of dishes for innoculation of transduction mixture and on volume of innoculating mixture on one

dish, optimal 100–300 µl (0,03 M stock solution of sodium citrate – add to transduction mixture in ratio 1:1; 0,017 M stock solutin for resuspendation of pellet).

- Innoculate the transduction mixture on dishes with MPA enriched by sodium citrate (20 mM) and tetracycline, 5 µg/ml (1 M stock solution of sodium citrate – add 2 ml in 100 ml of boiled sterile medium. Stock solutioon of tetracycline, 5 mg/ml –add 1/1000 of volume in boiled sterile medium).
- Incubate the dishes at 37 °C for 24 hours.
- Assess the number of colonies of transduction particles on dishes. Calculation of frequency of transduction is the quotient of mu,ber of transduction particles (CFU/ml – colony-forming units / 1 ml) to number of phage particles transduction lysate (PFU/ml – plaque-forming units / 1 ml).

Conclusion

Was the plasmid transferred? How did you the successful transfer verify?

Additional information

Rosypal S., Doškař J., Pantůček R., Kailerová J., Relichová J., Růžičková V., Šmarda J. ml., Šmarda J., Štěpán J., Terminologie molekulární biologie, MU Brno, 2001.

Varga M., Charakterizace systému nespecifické transdukce plazmidů u *Staphylococcus aureus*. Dizertation thesis, MU Brno, 2013.

Varga M., Transdukce plazmidů zprostředkovaná profágy kmenů *Staphylococcus aureus*. Diploma thesis, MU Brno, 2009.

Control questions

1. What we verify by controls?
    a. Dish with antibiotics + donor strain
    b. Dish with antibiotics + recipient strain before transduction
    c. Dish without antibiotics + recipient strain before transduction
2. Where (control dish) shloud the cells grow/not to grow?
3. What is the transduction?
4. Are the phages with lytic or lysogennis cycle used for the transduction, why?

Indirect assessment of number of viable bacteria by cultivation

Aim of practice

Dilution of cell suspension. Determination of viable cells number.

Introduction

Direct and indirect methods are used to assess number of cells in sample. **Direct methods** exploit microscope, counting of cells in preparation of sample without cultivation. Advanatage is to assess as live as dead cells or their ratio. **Indirect methods** are cultivations. Number of viable cells in sample is estimated by cultivation. Nephelometric analysis exploit the light intensity reflected from cells, it is determination of optical density of cell suspension. Advantage is to determinate standard curve, number of cells could be deducted from the liean part of curve. It is necessary to pay attention on cell lysis by osmotic shosk during dilution of sample. Number of dilution tubes is identified according to density of cell suspension. Suspension seems to be without opalescence at values 0 - 10^3 CFU/ml; has moderately opalescence at 10^5 CFU/ml; value 10^7 - 10^9 CFU/ml forms milk turbidity according to size and shape of cells.

It is necessary to know the exact cell number in sample for some physiological and genetic experiments. Cell number serves as standard to compare the results. Unit for calculation of assessment of cell number in sample by cultivation is number of viable cells (forming colonies on dishes) in 1ml of medium/sample, s.c. **colony forming units - CFU/ml**. The method is based on assessment of number of growed colonies supposing that one cell forms only one single isolated colony. Cultivation could be performed two ways: to mix the sample with liquid tempered agar and to pour the mixture on dishes or pipetting of sample on dish with agar and smearing of sample by sterile stick (fig. 21).

Fig. 21. Innoculation of cell suspension onto the dish – smearing by the bacteriological stick.

The number of cells (CFU/ml) or the turbidity of suspension (%) is always mentioned in protocols of experiments. Turbidity is less acurate value and serves only for quick orientation. Turbidity of medium or dilution solution containing dead cells could be measured in spectrophotometer. For experiment repeats, it shloud be maintain the same conditions (the same old culture, the same turbidity) to suppose the probably same number of dead and live cells in suspension.

The set of methods could be used to assess of cell numbers in various environments, the most suitable methods have to be choose for experiment purpose. Assessment of cell number is performed in certain volume and is mostly recalculated to 1 ml of original sample for correcting of experimental results.

Increasing number of cells could be observed during time by assessment of CFU/ml. If the appropriate mixed sample are assessed and evaluated from liquid medium during cultivation, very precise example of physiological state of culture (physical or chemical effects on growing cultures, supporting or inhibiting coumpound in food industry or in microbial technologies) could be evaluated by comparing of cell numbers in time. Growth curve of microorganisms could be constructed by graphic illustration of CFU/ml value (samples from liquid medium without any nutrients addition) taking in the same time intervals in certain environment. Ratio of individual bacterial groups could be evaluated from mixed culture or comparing of macroscopic markers of cultivated species, it is necessary to use as basic universal as selective or selective-diagnostical media.

S.c. automatic colonie counters (fig. 22A) or smearing and washing out methods by analyser (fig. 22B) could be use in industrail operations. Instrument Lumitester PD (fig. 22B) can evaluate the total level of contamination without species and genera differentiation (measurement of luminiscence) very quickly and is mostly used i.e. in food industry.

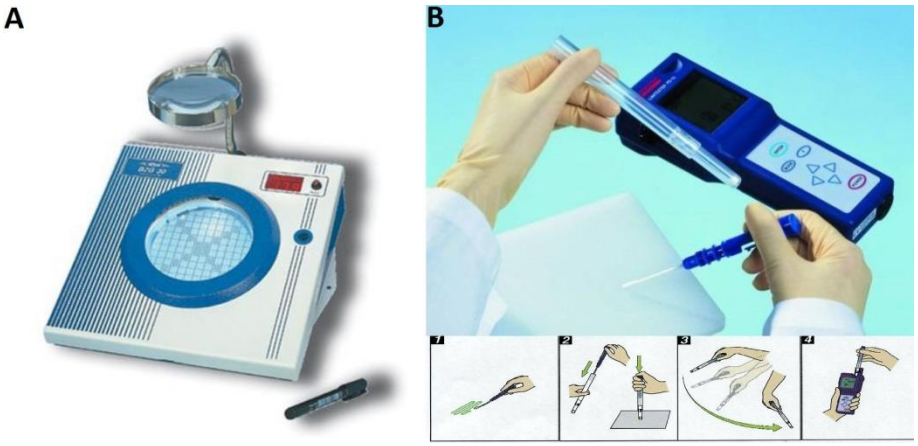


Fig. 22. Instruments for automatic colonies counting (A; http://www.marconi.sk/?katid=788, 1. 3. 2016, adjusted) and total level of contamination (B; http://qualifood.cz/index.php?route=product/product&product_id=56, 1. 3. 2016, adjusted).

Instruments and microorganisms

Sterile dishes with MPA

Sterile tubes, pipettes, sticks

Sterile phosphate buffer or physiological solution

Micrococcus luteus CCM 169

Methods (fig. 23)

Dilution of sample

- Pipette 0,9 ml of sterile physiological of phosphate buffer in to set of tubes.
- Pipette 0,1 ml of mixed sample suspension in to first tube with buffer.
- Mix the sample (total volume 1 ml), pipette 0,1 ml of mixed sample in to second tube.
- Mix the sample, pipette 1 ml in to third tube. Continue until the end of dilution set.

Innoculation

- Sign the Petri dishes with MPA on the lid (colonies will be counted by dotting on the bottom of the dish by the felt-tip).
- Pipette 0,1 ml on the dishes by the dilution from 10^{-4} to 10^{-6}. Use at leat 2 dishes for every dilution for calculation of average number of colonies from target dilution.
- Smear the suspension by sterile stick all over the agar, turn the dish against the smearing, do not push on stick. Open the dishes as little as posibble. Smear the sample uniformly for isolated grotwth of colonies.
- The cultivation is performed for 2-3 days at 30 °C.

Control of dilution buffer sterility

- Pipette 0,1 ml of physiological buffer on dish and smear it by the stick. The cultivation is performed for 2-3 days at 30 °C.

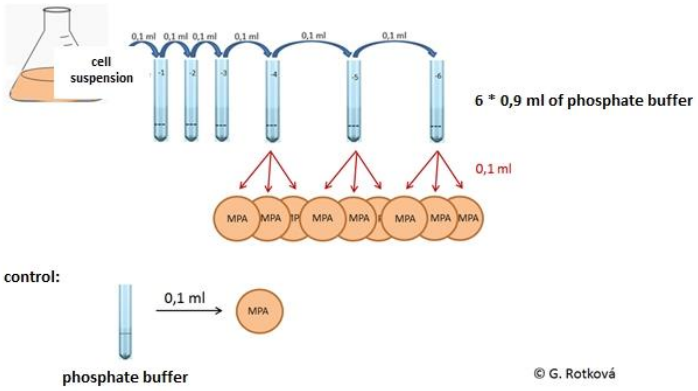


Fig. 23. Scheme - procedure of assessment of cell number by cultivation.

Evaluation

Choose the pair of dishes of suitable dilution (fig. 24, dishes with circa 20 - 200 colonies) and count the number of colonies. Count the colonies from bottom of dish and dot every colony by felt-tip on glass. Count the average of number of colonies, multiply the average by the positive value of dilution and multiply the number by 10 (only 0,1 ml of sample was pipetted on dish, the results is recounted on 1 ml) to get value of **CFU/ml** (colony forming units, number of cells forming one colony in 1 ml).

CFU/ml = average number of colonies * value of dilution (positive exponent) * 10

Right format of result is i.e. $2{,}4*10^4$ CFU/ml, not the $24*10^3$ CFU/ml.

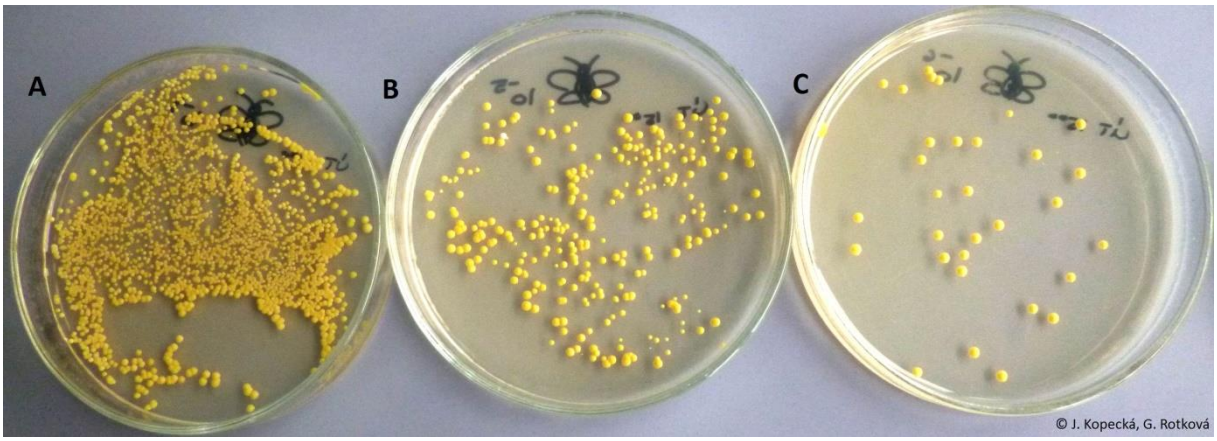


Fig. 24. Assessment of cell number by cultivation. Appropriate dilution for counting of colonies is dilution 10^{-6} (C) in this case, dilutions 10^{-4} (A) and 10^{-5} (B) contain too many colonies.

Conclusion

What dilution (number of colonies) was the most suitable for reading of results? What number cells (CFU/ml) was in the original sample? Did you work aseptically? If not, why? Was recorded the difference between number of colonies of one dilution (nonmixed sample)? Did bacteria grow on control dishes with buffer only? Could the contamination affected the total number of CFU/ml, why (morphotype of colonies)? Was the sample good countable?

Additional information

Klaban V., Ilustrovaný mikrobiologický slovník, Galén, Praha, 2005, ISBN 80-7262-341-9.

Control questions

1. What is the calculation of CFU/ml and what individual constituents of calculation means?
2. In practice, we used nonselective medium and pure culture of *Micrococcus*. In what case are used the selective media?
3. If we use i.e. pond water instead of cell suspension of *Micrococcus*, how and why shloud be changed the morphology of growing bacterial colonies? Will colonies from pond water of single morphological type?
4. According to what we consider number of tubes with buffer (number of dilutions), when we use only one or two tubes and when we use higher number of dilutions?
5. What unit is used for calculation of number of cells assessed by cultivation?
6. Why and how is the control of dilution solution performed?
7. What means the unit CFU/ml?
8. Why is not used innoculating loop?
9. Calculate CFU/ml, if number of colonies is 210 and 190 in dilution 10^{-6}. 0,1 ml of culture was transferred into the 0,9 ml of buffer for dilution of suspension.
10. Desribe the nature of assessment of cell number by cultivation.
11. What is the difference betweendirect and indirect estimation of cell number?
12. Why is essential mix the sample before transffering?
13. How we recognize th contamination of dilution solution?
14. Why is innoculated only 0,1 ml and not 1 ml on the dish?
15. According to what is choose the appropriate type of nutrient medium?

Direct assessment – number of cells in Bürker chamber, vital test, yeasts

Aim of practice

Determine the percent of surviving cells depending on time of incubation in higher temperature.

Introduction

Direct and indirect methods are used for determination of number of cells in sample. **Indirect methods** are cultivation methods. **Direct methods** exploit microscope, counting of cells in preparation of sample without cultivation. Advantages of direct methods are speed, possibility to differentiate live and dead cells, determination of their ratio.

It is necessary to have suspension with appropriate cell density for counting of cells in preparation. Stained preparations are used for dead and live cells differentiation. Cells could be counted by fixed stained or nonstained preparation, live and dead cells are not differentiated in this case. Counting chambers are used for direct assesment of cell number, i.e. Thoma, Bürker chamber (figr. 25), chamber is glass plate with counting grating of various size. Space between microscopic slide and cover glass is marked on chamber. Defined volume of sample in chamber is recounted to 1 ml of sample. Cells are settled down and could be counted on certain area/fields of chamber.

Fig. 25. Bürker counting chamber.

Vital test (staining of native preparation) serves for evaluation of current state of cell population. It is based on permeability of dead cell membrane. Plasma membrane of dead cells is not semipermeabila, dye is getting inside the cells. Live cells defend to permeation of dye by membrane channels; dye is excluded or degraded. Nontoxic dyes are used to staining of dead cells, i.e. methylene blue solution diluted und buffered by phosphate (pH 4,6). Effect

of increased temperature on surviving of cells in time could be observed by vital test. Advantages of test are speed, low material demand compared to cultivation methods, posibility to differentiate ratio of live and dead cells. However, observed cells can not be cultivated after that.

Vital test is used to control of microbial vitality during the technological processes. Their answear (change in number or ratio) to addition or decline of various compounds during process could be observed in time. On the base of results, technological process during cell cultivation could be regulated.

Yeasts belong among heterothrophic **eukaryotic** organisms, fungi or micromycetes together with moulds. Yeasts are able to ferment mono-, di- or trisaccharides to ethanol and CO_2. Yeast are mostly unicellular organisms, but could form mycelia or pseudomycelia sometimes. Pseudomycelium originates by budding of cells on poles, single cells are not separated, stay connected. Filament of pseudomycelium is composed from elongated cells. Hyphae of mycelium are separated by septa, mycelium has the same diameter over the whole length. Yeasts reproduce by budding or fission; form colonies bigger than bacteria and ascospores on solid media. Most of yeasts has low temperature resistance, they are killed by 2-5 minutes of warming at 56 °C. Spores are more resistant. Cell wall is stained as grampositive, but the composition is quite different from bacterial cell wall. Cell wall of yeast does not contain peptidoglycan, but comprises from glucana, mannans, proteins and chitin. Cell cycle is divided to G1, S, G2 and M phases in yeast compared to only S and D phase in bacteria. Life cycle of yeast is composed from haploid (spores) and diploid phase that could be supressed in profit of the other phase. More than 1 spore (mostly multiple of 4) is originated in yeast cells.

Yeasts are widely used in food industry (bakery, beer, wine), to ethanol and recombinant proteins (recombinant vaccine - hepatitis type B) production and as model organism. Pathogenic yeasts are i.e. *Candida, Cryptococcus, Malassezia* and *Trichosporon.*

Saccharomyces cerevisiae is the first eukaryotic organism with whole genome sequenced (year 1996), occurs commonly in environment, is used especially in food industry and for research.

Candida belons among the most ordinary yeast infection agents. Nonpathogenic form has cells in yeast unicellular form (ovoid, single cells). If *Candida* causes disease in organism, it switches to myceliar form of growth.

Schizosaccharomyces pombe is one of a few special yeast species that is reproduced by fission. Its genome contains only 3 big chromosomes.

Instruments and microorganisms

Yeasts *Saccharomyces cerevisiae*, bakery yeasts

Erlenmeyer flask, tubes, pipettes, dropper

Sterile distilled water

Water bath, thermometer, microscope

Bürker chamber

Methylene blue

Methods

- Prepare the suspension of yeast cells from bakery yeast or from culture yeast in sterile distilled water in flask.
- Pipette 1 ml of suspension in to 4 tubes.
- First tube serves as control sample to determinate number of live and dead cells on the start of experiment (0 minutes).
- Assess the number of live and dead cells. Pipette the drop of the suspension in Bürker chamber, cover it by the cover glass. Drop the methylene blue to the edge of cover glass. Put the filter paper to the oppposite edge of cover glass and let it absorb the suspension until the whole preparation is stained by methylene blue.
- Observe the preparation after the setting the cells (1-2 minutes), count the colourlees live and dead (blue) cells at magnification 400x.
- Count the cells for every time at least in 10 fields. Include in to the sum of cell also the cells laying on 2 edges of filed (right and upper edge or left and bottom edge, fig. 26)

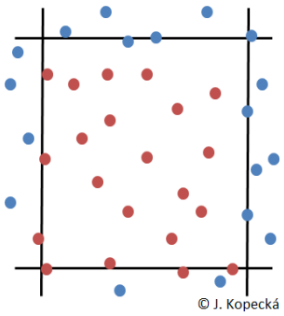


Fig. 26. Counting of cells. Red cells are included in to the total number of cells.

- Place the remaining tubes in the water bath at 61 °C. Assess the number of live and dead cells after 7, 14 and 21 minutes (fig. 27) by the method described above.

- Calculate the total number of cells (dead + live).
- Make the graph of relationship - surviving cells and time of increased temperature treatment (axis x, % of surviving cells; axis y, time).

$$\text{total number of cells/ml} = \frac{\text{sum of live and dead cells}}{10\ (\text{number of fileds})} * 250 * 1000$$

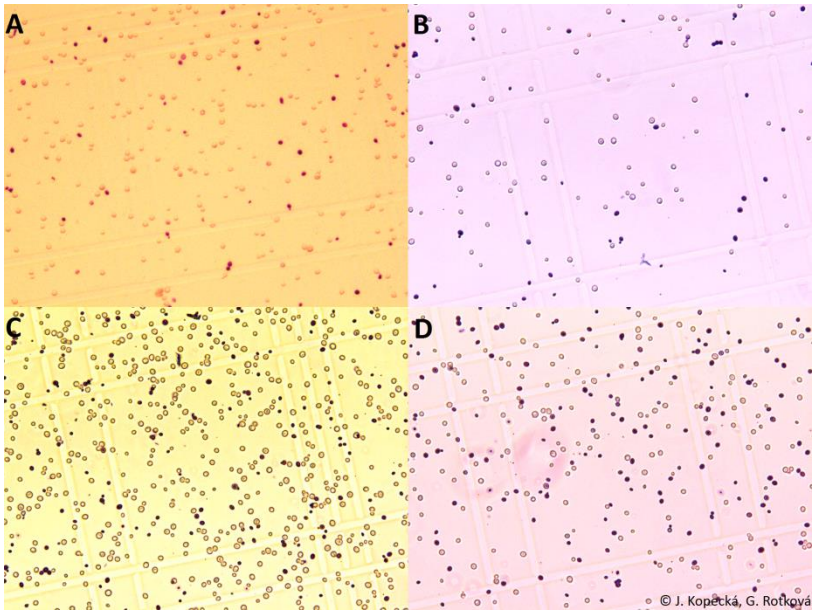


Fig. 27. Stained cells in vital test in time 0 (A), 7th (B), 14th (C) and 21th minutes (D).

Conclusion

Did the number of live cell continuously decrease? Were the live and dead cells good distinguishable from each other? In what time was he biggest decline of live cells monitored?

Additional information

Klaban V., Ilustrovaný mikrobiologický slovník, Galén, Praha, 2005, ISBN 80-7262-341-9.

Control questions

1. What is the principle of the vital test?
2. How we determine the actual state of cell population with regard to the ratio of live and dead cells?
3. What is the procedure for observing of effect of physical or chemical factor in time on ratio of live and dead cells in population?

4. Do we estimate via vital test the total number of cells in suspension?
5. With what aids we work during the direct estimation of cell number?
6. Desribe the nature of cell counting in Bürker chamber.
7. Do we determine number of live or dead cells via vital test?
8. How we differentiate live and dead cells?
9. What physical characteristic is observed during the vital test?
10. For what are important the time intervals during the vital test?

AP-test (test of acidification power)

Aim of practice

Determine of physiological condition of yeast due to the AP-test.

Introduction

Physiological state and metabolic competence of pitching yeasts is the key factor affeting the quality of final product during the beer production. Viability (number of live and dead cells in culture) and vitality (fermentative and metabolic capability of cells) of yeast cells play important role during beer production. Vitality is decreased in older cells or by stress response. New types of stress could arise from introduction of modern technologies (high hydrostatic pressure in cylindroconical tanks) or new raw materials. Set of methods was developed for assessment of yeast viability and vitality. Methods are based on vital staining, assessment of reproducing ability of cells, measurement of important compounds content, assessment of important enzyme aktivity, assessment of metabolic speed of cells, assessment of energetic state of cells. Methods have advantages and also limits. Optimum assessment of pitching yeast cell condition during the propagation as well as during fermentation, storage and especially for prediction of behaviour during the next fermentations comprise the combination usage of several techniques. It requires various and expensive instrumental equipments.

AP-test is used for assessment of metabolic competence of brewing yeasts that are pitched repeatedly, is based on knowledge of membrane procession in yeast that metabolize endogenic and exogenic substrates. The most important procession for acidification ability of yeast, activity of H+-ATPase, depend on cell condition (fresh cells, storage, type of cell washing) nad on growth phase. Activity of H+-ATPase in *S. cerevisiae* is strongly decreased during the diauxic-passage and stay low after post-diauxic and stationary phase. It is connected with switching to energysaving state and to minimalize energeticallydemanding processions with high utilisation of ATP.

The principle of AP-test is measurements of environmental change (pH value) induced by yeasts. Change of pH value of extracelular environment is measured for 10 minutes, followed by adding of glucose and by measurements for another 10 minutes. PH of yeast suspension decreases significantly after addition of sugar. Decrease of pH value is caused by formation of electrochemical gradient of protons through plasma membrane by nutrient absorption.

Gradient of pH is formed by membrane ATPase. Except for ATPase, elimination of metabolic products, mostly weak acids in environemnt, participates also on acidification capability of yeast. Yeast cell exploits energy of storage polysaccharides, glycogen and trehalose, for maintaining of ration extracelular and intracelular pH at deficiency of metabolised sugars. Cells use endogenic and exogenic metabolism to maintain the homeostasis in sugars presence. Change of pH value in first 10 minutes after adding of yeast in water (absence of outer source of glucose) is called spontaneous acidification ability that expresses the growth ability of yeasts and is related with storage polysaccharides content in cell. Another decline of pH valu eis measured after 10 minutes from adding the sugar in solution. Glucose induced acidification ability showes the activity of yeasts during fermentation and the speed of glycolysis. Acurately measurements of pH value and right calibration of pH meter is basic for reproducibility of results. Variation in weight of cells ± 11 % does not affect the results at maintaining pH meter right conditions. The dry mas sis not needed to estimate.

Instruments and microorganisms

Yeasts: dried compressed yeasts or weighing bakery yeasts, brewing yeasts
Distilled water, centrifuge, pH meter, electromagnetic stirrer, solution of glucose (50 %)

Methods

Modification of AP-test according to Hollerová et al., 2005

- Weigh 9 ±0,1 g of yeasts, add 50 ml of cooled distilled water and wash the cells.
- Centrifugate the suspension (3000 x g/10 minutes), wash the cels. Repeat two times.
- Resuspend yeasts in 50 ml of distilled water at room temperature.
- Start the measurement of pH values (plunge pH meter into solution) – **time 0 (pH_0)**.
- Mix the sample on electromagnetic stirrer.
- Add 5 ml of glucose (50 % solution) after 10 minutes, **record the pH valuie (pH_{10})**.
- Record the **pH value in 20th minutes (pH_{20})**.

Calculation of AP value

AP_{10} value responds to result of spontaneos acidification before adding the glucose, it is calculated from pH value in 10th minutes of measurement (pH_{10}): $AP_{10} = 6{,}3 - pH_{10}$
where 6,3 is the value of pK system CO_2/HCO_3, approximately equals to intracelular pH value.

AP_{20} value responds to result of glucose induced acidification. I tis calculated from pH value in 20^{th} minutes (pH_{20}): $\boldsymbol{AP_{20} = 6{,}3 - pH_{20}}$

Final AP_{20} value reflects the physiological state of yeast cells:

AP_{20}	State of cells
>2,5	highly active yeast cells with strong fermentative capability
2,5 – 2,0	active yeast cells with good fermentative capability
2,0 – 1,5	partly damaged, cells with lower metabolic activity
< 1,5	damaged cells, not suitable for another pitching

Conclusion

What was the physiologic state of used yeasts? What was the result of vital staining compared to AP test?

Additional information

Gabriel P., Dienstbier M., Matoulková D., Kosař K., Sigler K., Optimised acidification power test of yeast vitality and its use in brewing practice. J. Inst. Brew., 2008, 114: 270–276.

Hollerová, I. a kol., Vitalita a viabilita násadních kvasnic: metody posuzování a vliv buněčných systémů pro stresovou resistenci. Kvasny Prum., 2005, 51:3-7.

Košin P., Šavel J., Kolouchová I., Brož A., Viabilita a vitalita kvasnic v provozním kvašení. Kvasny Prum., 2007, 53: 30-34.

Sigler K., Matoulková D., Stress responses in brewing yeast. Kvasny Prum., 2011, 57:277-284.

Šavel J., Měření aktivity pivovarských kvasinek. Kvasny Prum., 1999, 45:258-261.

Control questions

Which methods do you know for testing of yeast state?

Are yeasts with quick utilisation of added glucose during the AP test suitable for usage in technological process?

Are yeasts with slow utilisation of added glucose during the AP test suitable for usage in technological process?

What activity of yeasts is evaluated (pH value) in 10^{th} and 20^{th} minutes? Is there any difference between them?

Physical and chemical agents for control of microorganism growth

Aim of practice

Testing of effect target physical and chemical products for control of microbial growth depending on time of contact and concentration of products (UV radiation, disinfection compounds: SAVO, Incidur and Ajatin).

Introduction

Factors of environment could affect microorganisms **microbicidal** (irreversible inhibition with lethal effect) or **microbistatical** (reversibile effect, after factor i.e. dilution, cells continue in growth).

Inhibition effect is depend on character and intensity, physiological state of cells and on environment. Vegetative cells are more sensitive than static forms, spores and cysty. Sensitivity is influenced by cell and cell wall compositionky, by genus or species of organism. Organisms producing pigments are better protected form UV radiation. Dyes in pigments absorb certain wave-length of light that could be used as source of energy for nurients synthesis and another mechanisms.

Decontamination is removing of microbial, chemical and radioactive contamination from objects.

Antisepsis is disposal of germs in live tissue environment, in injuries, on mucosa and on skin by using of antiseptics. Antiseptics are as bactericidal as bacteriostatical. Antiseptics have to be nontoxic and suffered good by live tissue.

Asepsis is the set of principles leading to minimum of microorganisms in environment. Asepsis defends to entry of microorganisms to live tissue during surgery operation using of sterile instruments. Asepsis comprises also laboratory and production methods, i.e. microbiological laboratory, production of medicaments.

Disinfection is defined as destroying of contaminating microorganisms on abiotic objects in environment (water, air) and in infection materials. It include a broad scale of operations against to viruses, vegetative form of bacteria and fungi, not against to spores. Effectivity of disinfection depends on microbial resistance against to disinfection agents.

Sterilization is disposing of all live organisms in environment including viruses, bacteria, fungi, bacterial and fungal spores.

Physical methods of sterilization

Fractionated sterilization, tyndilization and sterilization by saturated water vapour belong to among sterilization by moist heat.

Interrupted, fractionated sterilization is sterilization by boiling (100 °C) for 30 minutes in 18-24 hours intervales in 3 days. Sterilized compound have to be deponated amongs intervats at room temperature for spores germination. Subsequent boiling kills it as vegetative forms of bacteria.

Tyndalizace is used to sterilize thermolabile protein solutions that could coagulate at 60 °C. Solution is warmed up in water bath at 56-58 °C (resp. at 60-80 °C) for 30-60 minutes in 3 days.

Sterilization by saturated water vapour under pressure (in autoclave) is used mostly at 100 kPa at 120 °C for 20-30 minutes. This process enable to destroy all forms of microorganisms. Autoclave is pressure sterilizer in that could be sterilized sling material, various solutions, metal medicinal instruments, rubber material. It is necessary to pay attention to possibility of hydrolysis of disaccharides and damage of thermolabile compounds.

Dry heat has less efectivity than vapour under pressure. It has lower coeficient of conduction, sterilization takes part at higher temperature and for long period. Open flame is used to singe of bacteriological loop, to liquidate laboratory animals and some insruments of low weight, i.e. contaminated bandages.

Hot-air sterilization of glass, porcelain and metals takes place in hot-air steriliser. Time is counting after achievment of target temperature, mostly at 160 °C for 60 minutes or at 180 °C for 20 minutes.

Sterilizace by filtration serves to elimination of microbes from liquids when the other ways of decontamination is not suitable. Viruses go through th most of bacterial filters. Filters differ according to construction, size of pores and material. Azbest Seitz filter are pressed from azbest and cellulose. Filters retaining bacteria are termed EK (Entkeimung). Filtration inserts are single-purpose and are sterilized with filter funnel in autoclave. Glass filetrs are composed from borosilicate glass in form of porous plates in funnels. It could be used repeatedly (washing with concentrated sulphuric or chrom-sulphuric). Membrane ultrafilters from nitrocellulose have various pore size and diameter.

Sterilization by radiation is carried out by UV or ionizing radiation.

Ultraviolet radiation (UV) has optimum bactericidal effect at wave-length around 254 nm, radiation is absorbed at maximum by nucleid acids. Germicidal lamps are used as sources. UV radiation serves to sterilize air, working area (operation room, aseptical boxes, dissecting

and bleeding rooms, sanatoria. Radiatin could not compensate the cleaning by disinfection agents. Effectivity of UV radiation decreases with distance to exposed object.

Ionizing radiation penetrates, do not warm sterilized object and do not change characteristics of most sterilized compounds. Source of gama radiation of mostly radioactive cobalt. Gama radiation is used to industrial sterilization (sling material, plastic). International determinated sterilization portion is 27 kGy.

Chemical disinfection

Sampling and microbiological evaluation of used disinfection solutions is tested on standardn microbial strains. Results are interprets with regard to specific reasons of control. Disinfection effectivity could be bactericidal, bacteriostatic, fungicidal, fungistatic, tuberculocidal, mycobactericidal, sporicidal, sporistatic, virucidal. Testing could be modified according to conditions – more or less clean/dirty environment. Specific effect of chemical compounds is displayed according to their concentration and time of exposition.

Criteria of disinfection agents quality: broad spectrum of effectivity (bactericidal, virucidal, fungicidal effect), do not originate resistance during long-term usage, nontoxic, quick disinfetion effect, affinity to microorganisms, inert to disinfected object, stable disinfection effect in various conditions (temperature, air humidity, pH).

Antimicrobial compounds damage mostly direct microbial structure or affect basic metabolic processes i.e. by oxidation (compounds of chlorine, peroxides, acid peroxides), by reduction (aldehydes), by hydrolysis (acids, hydroxides), by dehydratation (alcohols), by protein coagulation (alcohols, phenols), by change of permeability (detergent compounds).

Bases and acids. Strongly inorganic acids and bases are used very rarely for their toxic and aggressive effects, i.e. whitewash, boracic acid, peracetic acid, persteril (32-36 % solution of peracetic acid with 10 % H_2O_2 and 1 % H_2SO_4)

Oxidation compounds: hydrogen peroxide, potash

Halogens compounds: bleaching lime, Chloramin B, Dikonit

Iodine and its compounds: iodine tincture, triiodomethane, Jodonal B, Jodisol

Heavy metals compounds: Famosept, Merfen, Merthiolate, Thiomersal

Alcohols: ethanol, n-propanol, etylenoxide

Aldehydes: formaldehyde, formalin, glutaraldehyde

Phenol derivates: cresols, Lysol, Orthosan BF 12

Surface active agents: Ajatin, Septonex, Ophthalmo-Septonex

Set of tests is used to control effectivity of disinfection and sterilization according to character of sterilized compound and type of sterilization or disinfection, i.e. paper indicators or bioindicators, smears by sterile cotton-wool swabs.

Instruments and microorganisms

Petri dishes with MPA, tubes with 10 ml MPB
Sterile cotton-wool swabs, tubes, pincette, distilled water
paper, alluminium foil, pipettes
UV lamp
Dissinfection agents (Savo, Incidur, Ajatin)
Pseudomonas fluorescens
Staphylococcus aureus
Escherichia coli
Saccharomyces cerevisiae
Serratia marcescens
Bacillus cereus

Methods

Impact of exposition time of UV radiation on microbial growth

- Smear the microbial culture over the whole agar in sufficient density by the cotton-wool swab (3 dishes with MPA).
- Divide the dishes from the bottom by the felt-tip to halfs. Place the dishes in the box with UV lamp and take off the lid of dish. One half of dish cover with alluminium foil.
- Expose first dish for 10 seconds, second dish for 30 seconds, third dish for 60 seconds to UV radiation.
- Cover the dishes after radiation by the lid. Incubate for 24 hours at 30 °C.

Evaluation: Read the growth of bacteria on dishes, record the result. Evaluate the impact of UV radiation on bacterial growth according to time of radiation. Evaluate bacterial growth in coverred part of dish (control). At what time of radiation is observable decreasing of bacterial growth? Were microorganisms affected by UV radiation? Did the effect depend on microbial species/genus and on the time exposition?

Impact of exposition time on microbial growth

- Prepare the disinfection agent in concentration recomended by the manufacturer in sterile tube
    - Incidur: 0,5% solution, total volume 5 ml, 25 µl of agent + 4,975 ml of water
    - Savo: 100 ml in 3 l, 3,33% solution, pipette 166,7 µl of Savo in 4,833 ml of water
    - Ajatin: 1% solution
- Divide the dish with MPA in 3 sectors from bottom by felt-tip, sign sectors 0; 1 and 10 minutes.
- Innoculate culture by cotton-wool swab by „snake-motion“ in the sector 0 (control).
- Add 500 µl of culture in tube with disinfection agent and mix it.
- Innoculate culture by cotton-wool swab by „snake-motion“ after 1 and 10 minutes to the target sector on dish.
- Incubate for 24 hours at 37 °C.

Evaluation: Read the growth of bacteria on dishes, record the result. Evaluate the effect of exposition time of certain disinfection agent on bacterial growth. Compare the density of growth in various exposition times. Affect the exposotion time of disinfection agent microbial growth?

Effect of concentration on microbial growth, assessment of minimum inhibition concentration

- Sign sterile tubes with number 1 to 6.
- Prepare 2% solution of Incidur or 3% solution of Savo in MPB in total volme of 2 ml (40 µl of Incidur + 1,960 ml of MPB; 60 µl Savo + 1,940 ml of MPB) in the tube number 1.
- Pipette 1 ml of MPB in another 5 tubes.
- Pipette 1 ml of solution from the first tube to the second tube and mix it.
- Repeat the procedure until tube number 5. Pipette 1 ml of solution from fifth tube to infection waste.
- Last tube (6) contains no disinfection agent, only the MPB (control of growth). Tubes 1 to 5 contain 1 ml of disinfection agent solution with decreasing concentration in MPB. Concentration of agent is half-value in each following tube compared to previous tube.
- Innoculate 50 µl of cultures in every tube. Incubate for 24 hours at 37 °C.

Evaluation: Compare the growth in various concentrations of disinfection agent. What concentration is sufficient to kill target cultures? Is affected the minimum inhibition concentration of disinfection according to bacterial species and type of disinfection?

Conclusion

Results of experiments are shown in fig. 28 and 29.

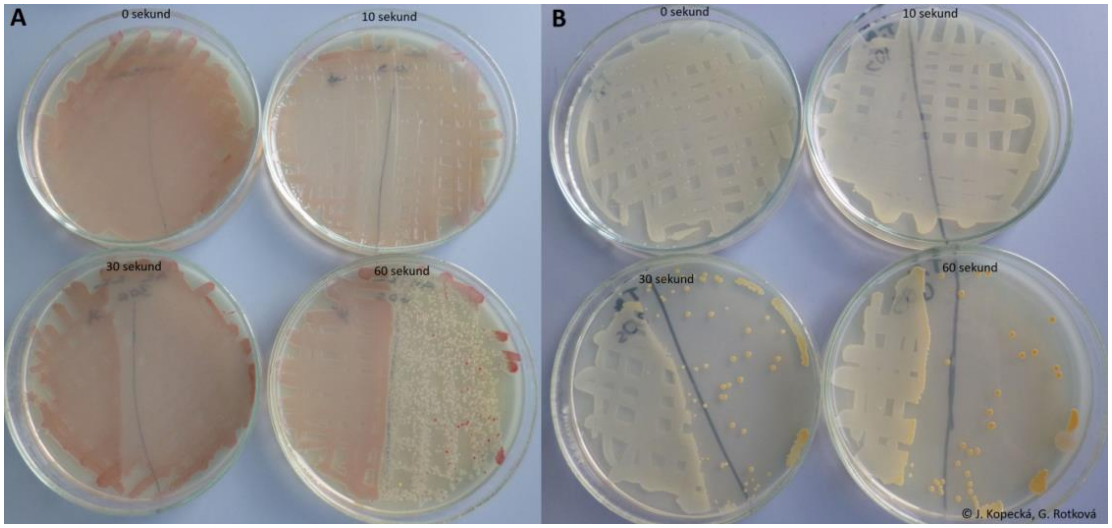


Fig. 28. Effect of UV radiation on growth *Serratia marcescens* (A) and *Staphylococcus aureus* (B).

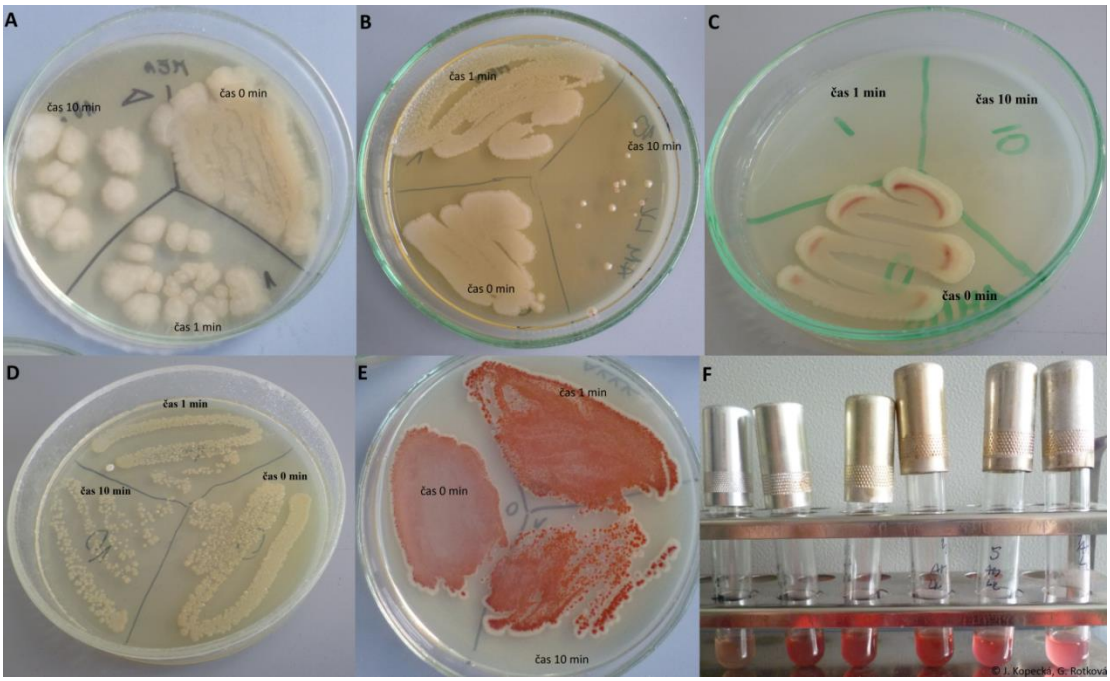


Fig. 29. Influence of different time of affecting of disinfection agents on growth of *B. cereus* (A), *S. cerevisiae* (B), *S. marcescens* (C), *E. coli* (D), *S. marcescens* (E) and influence of various concentration of disinfection agents on growth of *S. marcescens* (F).

Additional information

Kneiflová J., Hodnocení baktericidní účinnosti dezinfekčních přípravků suspenzní mikrometodou. Čs. epidemiol. 1988, 37:97–103.

Standardní metody pro hodnocení dezinfekční účinnosti chemických látek. AHEM, příloha č. 1, 1985, 1–25.

Melicherčíková V., Sterilizace a dezinfekce ve zdravotnictví. Praha, Grada Publishing, 1998.

Control questions

1. Why is necessary remove the glass lid of Petri dish during UV radiation of agar?
2. Many microorganisms have pigments in nature. What advantages brings pigment for organism?
3. On what depends the efficiency of physical and chemical agents with respect to the elimination of microorganisms?
4. Desribe the term microbistatic.
5. Desribe the term microbicidal.
6. Does the UV radiation go throught the aluminium foil?
7. Where is used the sterilisation with UV radiation?

Effect of target dyes and alcoholic beverages on bacterial growth

Aim of practice

Determine the sensitivity of bacteria to dyes and alcoholic beverages. Compare the effect of compounds according to changes of bacterial growth (inhibition of growth, change of pigmentation).

Introduction

Some organic dyes (Tab. 2 and 3) have bacteriostatic effect on grampositive bacteria in concentration that gramnegative bacteria still grow.

This attibute is used pro selective media preparation i.e. detection of coliform bacteria. Crystal or gentian-violet, malachite green, methylene blue, acriflavine are commonly added to media. Selective agents for streptococci are i.e. acridine orange, ethyl violet, aniline and trypan blue.

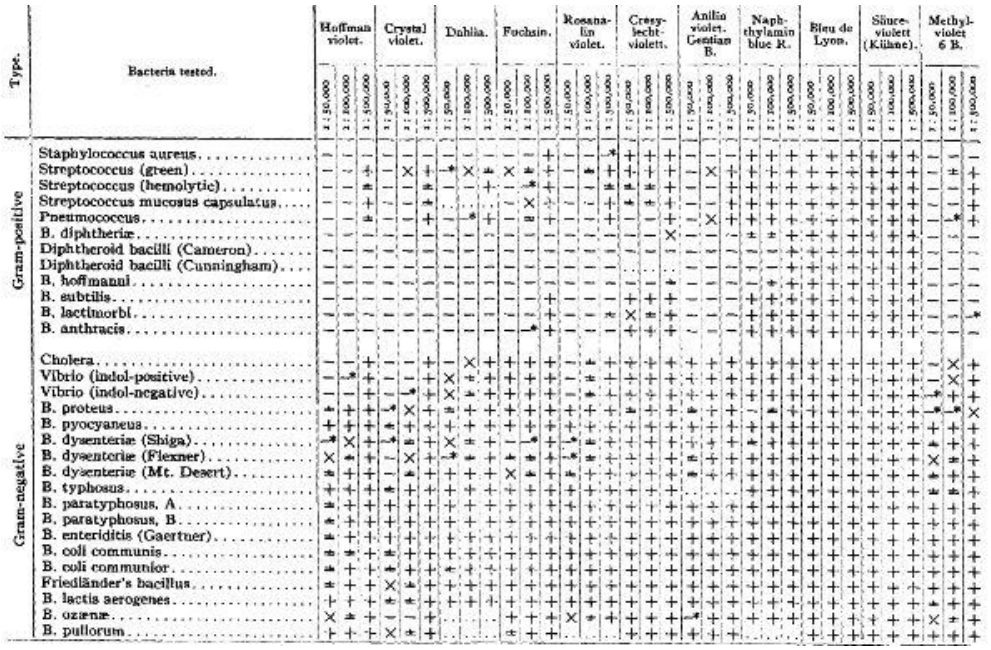
Tab. 2. Effect of basic, acid and neutral dyes at 1:1000 dilution on bacterial growth (Fung and Miller, 1973, adjusted).

Organism	Basic dye medium																			
	Janus Green	Methylene blue	Safranin O	Safranin Y	Basic fuchsin	Methyl green	Nile blue A	*p*-Rosaniline	Malachite green	Bismarck brown	Thionin	Thioflavine TG	Chrysoidine Y	Brilliant cresyl blue	Brilliant Green	Neutral red	Methyl violet B	Auromin O	Acriflavine	Crystal violet
Gram-negative organisms	Positive	Positive	Positive	Positive			Positive		Positive	Positive						Positive		Positive		
Alcaligenes faecalis					+	+		+			v	−	v	−	−		−		−	−
Enterobacter aerogenes 11a					+	+		+			+	+	+	+	+		+		+	+
Enterobacter aerogenes 11b					+	+		+			+	+	+	+	−		+		−	+
Enterobacter cloacae					+	+		+			+	−	v	+	+		+		−	+
Escherichia coli					+	+		+			+	v	+	+	−		−		−	−
Proteus vulgaris					+	−		−			v	−	−	−	−		−		−	−
Pseudomonas aeruginosa					+	+		+			+	+	+	+	+		+		+	−
Salmonella typhimurium					+	+		+			+	v	v	−	−		−		−	−
Salmonella typhosa					+	+		+			+	−	v	−	−		−		−	−
Salmonella paratyphi					+	+		−			v	+	+	+	−		−		−	−
Salmonella pullorum					+	+		+			v	−	v	−	−		−		−	−
Salmonella thompson					+	+		+			−	+	+	+	−		+		−	−
Shigella flexneri					−	−		−			−	−	v	−	−		−		−	−
Serratia marcescens					+	+		+			−	+	+	+	+		+		−	−
Gram-positive organisms	Negative	Negative	Negative	Negative	Negative	Negative		Negative				Negative	Negative	Negative	Negative		Negative		Negative	Negative
Bacillus cereus							−		−	+	v					+		+		
Bacillus danicus							−		v	+	+					+		+		
Bacillus polymyxa							+		v	+	+					−		+		
Bacillus subtilis							−		−	v	−					+		+		
Bacillus sulfidus							−		−	v	−					+		+		
Gaffkya tetragena							−		−	v	−					+		+		
Micrococcus rhodochrous							−		−	−	−					−		−		
Sarcina lutea							−		−	−	−					−		−		
Staphylococcus aureus 241b							−		−	−	v					+		+		
Staphylococcus aureus 241c							−		−	v	−					+		+		
Staphylococcus aureus 241f							−		−	v	v					+		+		
Staphylococcus aureus 241g							−			v	v					+		+		
Streptococcus bovis							−		v	v	−					−		+		
Streptococcus faecalis							+		v	+	−					+		+		
Streptococcus lactis							−		−	−	−					v		+		
Streptococcus liquefaciens							+		v	+	−					v		+		

Organism	Acid dye medium																			Neutral dye medium		
	Brom thymol blue	*o*-Cresolphthalein	Eosine B	Chlorophenol red	Rose bengal	Brom cresol purple	Erythrosine B	Eosine Y	Cresol red	Phenol red	Methyl red	Resazurin	Trypan blue	Brom phenol blue	Nigrosine	Aniline blue WS	Acid fuchsin	Indigo tetrasulfonate	Thymolphthalein	Wright's stain	Jenners stain	Carmine
Gram-negative organisms	Positive	Positive	Positive				Positive	Positive		Positive		Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Alcaligenes faecalis				−	+	+			+		v											
Enterobacter aerogenes 11a				+	+	+			+		+											
Enterobacter aerogenes 11b				+	+	+			+		+											
Enterobacter cloacae				+	+	+			+		+											
Escherichia coli				+	+	+			+		v											
Proteus vulgaris				+	−	v			+		−											
Pseudomonas aeruginosa				+	+	+			+		+											
Salmonella typhimurium				+	+	+			+		+											
Salmonella typhosa				+	+	+			+		−											
Salmonella paratyphi				+	+	+			+		−											
Salmonella pullorum				+	+	+			+		+											
Salmonella thompson				+	+	+			+		+											
Shigella flexneri				+	+	+			v		−											
Serratia mercescens				+	+	+			+		+											
Gram-positive organisms	Negative	Negative		Negative	Negative						Negative						Positive	Positive	Positive			
Bacillus cereus			−			−	−	v	v	−		+	+	+	+	+				−	+	+
Bacillus danicus			−			−	−	−	v	−		+	+	+	+	+				−	+	+
Bacillus polymyxa			−			−	−	−	v	+		+	v	+	+	+				v	+	+
Bacillus subtilis			−			−	−	−	v	−		−	v	+	+	+				−	+	+
Bacillus sulfidus			−			−	v	−	v	−		−	+	+	+	+				−	+	+
Gaffkya tetragena			−			−	−	−	v	+		−	+	+	+	+				−	−	+
Micrococcus rhodochrous			−			−	−	−	−	−		−	v	−	−	−				−	−	−
Sarcina lutea			−			−	−	−	v	−		−	v	−	−	+				−	−	+
Staphylococcus aureus 241b			−			−	−	−	v	−		+	+	+	+	+				−	+	+
Staphylococcus aureus 241c			−			−	−	v	v	−		+	+	+	+	+				+	+	+
Staphylococcus aureus 241f			−			v	−	v	v	v		+	+	+	+	+				−	+	+
Staphylococcus aureus 241g			+			−	−	v	+	v		+	+	+	+	+				v	+	+
Streptococcus bovis			−			−	−	v	+	v		−	+	+	+	+				−	−	+
Streptococcus faecalis			−			v	v	−	v	v		+	+	+	+	+				v	−	+
Streptococcus lactis			−			−	−	v	v	−		−	v	+	v	+				−	−	+
Streptococcus liquefaciens			−			v	v	−	v	+		+	+	+	+	+				v	−	+

Negative (-), positive (+), variable (v) growth.

Tab. 3. Effect of anillin dyes on bacterial growth (Krumwiede and Pratt, 1914a, adjusted).



Type.	Bacteria tested.	Hoffman violet.			Crystal violet.			Dahlia.			Fuchsin.			Rosanalin violet.			Cresylecht-violett.			Anilin violet. Gentian B.			Naphthylamin blue R.			Bleu de Lyon.			Säure-violett (Kiliani).			Methyl-violet 6 B.		
		1:50,000	1:100,000	1:500,000	1:50,000	1:100,000	1:500,000	1:50,000	1:100,000	1:500,000	1:50,000	1:100,000	1:500,000	1:50,000	1:100,000	1:500,000	1:50,000	1:100,000	1:500,000	1:50,000	1:100,000	1:500,000	1:50,000	1:100,000	1:500,000	1:50,000	1:100,000	1:500,000	1:50,000	1:100,000	1:500,000	1:50,000	1:100,000	1:500,000
Gram-positive	Staphylococcus aureus	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	*	+	+	−	×	+	+	+	+	+	+	+	+	−	−	−
	Streptococcus (green)	−	−	+	−	×	+	−*	×	±	×	±	+	−	±	+	+	+	+	−	×	+	+	+	+	+	+	+	+	−	±	+
	Streptococcus (hemolytic)	−	−	±	−	−	±	−	−	+*	−	−*	+	−	−	+	±	±	+	−	−	+	+	+	+	+	+	+	+	−	−	+
	Streptococcus mucosus capsulatus	−	−	+	−	−	+	.	.	.	−	×	+	−	−	+	+	±	+	−	−	+	+	+	+	+	+	+	+	−	−	+
	Pneumococcus	−	−	±	−	−	+	−	−*	+	−	±	+	−	−	+	−	−	+	−	×	+	+	+	+	+	+	+	+	−	−*	+
	B. diphtheriæ	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	×	−	−	−	±	±	+	+	+	+	+	−	−	−
	Diphtheroid bacilli (Cameron)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−
	Diphtheroid bacilli (Cunningham)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	.	.	.	−	−	−	−	−	+	+	+	+	+	−	−	−
	B. hoffmanni	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*	−	−	−	−	±	+	+	+	+	+	−	−	−
	B. subtilis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−	−
	B. lactimorbi	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*	×	±	+	−	−	−	+	+	+	+	+	+	+	−	−	*
	B. anthracis	−	−	−	−	−	−	−	−	−*	−	−	+	−	−	−	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−	−
Gram-negative	Cholera	−	−	+	−	−	+	−	×	+	+	+	+	−	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	×	+
	Vibrio (indol-positive)	−	−*	+	−	−	+	×	±	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	×	+
	Vibrio (indol-negative)	−	−	+	−	−*	+	×	±	+	+	+	+	−	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−*	+	+
	B. proteus	±	+	+	−*	×	+	±	+	+	+	+	+	+	+	+	±	+	+	±	+	+	−	±	+	+	+	+	+	−*	−*	×
	B. pyocyaneus	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. dysenteriæ (Shiga)	−*	×	+	−*	±	+	×	±	+	−	−*	+	−*	±	+	+	+	+	+	±	+	±	+	+	+	+	+	+	±	+	+
	B. dysenteriæ (Flexner)	×	±	+	−	×	+	−*	±	+	±	±	+	−*	±	+	+	+	+	+	±	+	+	+	+	+	+	+	+	×	±	+
	B. dysenteriæ (Mt. Desert)	±	+	+	−	±	+	+	+	+	×	±	+	±	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+	±	+	+
	B. typhosus	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+
	B. paratyphosus, A	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. paratyphosus, B	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. enteriditis (Gaertner)	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. coli communis	±	±	±	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. coli communior	±	+	+	±	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Friedländer's bacillus	±	+	+	×	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. lactis aerogenes	+	+	+	±	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B. ozænæ	×	±	+	−	−	+	.	.	.	+	+	+	×	±	+	+	+	+	−*	+	+	+	+	+	+	+	+	×	±	+
	B. pullorum	+	+	+	×	±	+	.	.	.	±	+	+	.	.	.	+	+	+	+	+	+	.	.	.	+	+	+	+	+	+	+	+

Growth like control (+), restrained growth (±), markedly restrained growth (x), no growth (-), some growth developed later (*).

Only specific microorganisms grow on selective media, the others are eliminated. It is advantage of certain group of microorganisms that could be covered or overgrowed by others. Selective media are composed from nutrient base and inhibitor of growth - chemicals (compounds of tellur, selenium, lithium), basic dyes, compounds decreasing surface tension (bile, salts of bile acids, sodium deoxycholate), selective toxic compounds (sodium azide), antibiotics. Unwanted microflora could be inhibited by acid or basic character of media or its increased osmolality (high concentration of salts or saccharides).

Different types of microbial cell wall lead to various sensitivity of microbes to target dyes or chemical compounds. Composition of cell wall is described in chapter Macroscopic and microscopic observation of microorganisms (page XX).

Alcoholic beverages contain compounds that eliminate growth and reproduction of microorganisms. Wine contains tannins and flavonoids. Microbiological stability of wine is affected by sulphuration of wine and higher amount of alcohol. Spirits contain high level of alcohol (over 20 %). Beer has low pH and alcohol content is relatively low (0,5-8 %). Hop and its compounds (humulons, lupulons, hop essences, tannins) play the key role in microbiological stability of beer.

Instruments and microorganisms

1% of water solution of crystal violet

sterile tubes, distilled water, pipettes, Petri dishes

tubes with 18 ml MPA

beer, hop extracts, wine, spirits

Bacillus subtilis CCM 2216

Serratia marcescens CCM 303

Escherichia coli CCM 3954

Micrococcus luteus CCM 169

Methods

Bacteriostatic effects of crystal violet solution

- Dilute the crystal violet solution in ratio 1:10, 1:100 and 1:1000.
- Heat up 4 tubes with 18 ml of MPA in water bath.
- Pipette each dilution of dye solution separately into 4 Petri dishes. Pour the each dish with 18 ml of MPA and mix gently.
- Divide the dish from bottom to four parts by felt-tip after solidification. Innoculate bacteria in the sectors.
- Incubate dishes at 37 °C for 48 hours.
- Read the growth of bacteria (fig. 30) and write results in the table. Compare the growth of gramnegative and grampositive bacteria.

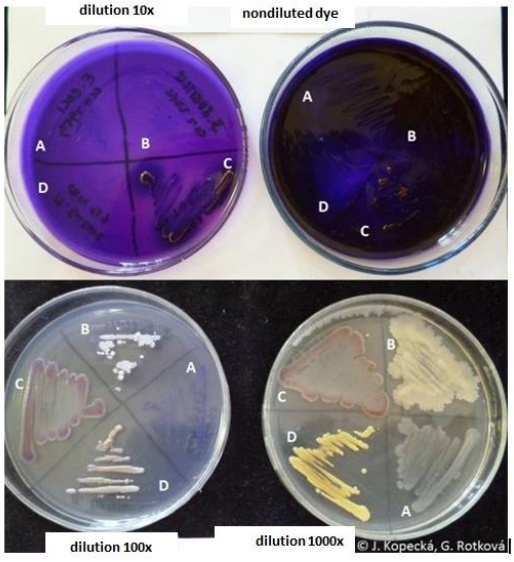


Fig. 30. Effect of crystal violet on microbial growth. *Escherichia coli* CCM 3954 (A), *Bacillus subtilis* CCM 2216 (B), *Serratia marcescens* CCM 303 (C), *Micrococcus luteus* CCM 169 (D).

Effect of beverages on growth of bacteria

- Heat up 4 tubes with 18 ml of MPA in water bath.
- Pipette 1 ml of each beverage (beer, wine, spirits, hop extract) separately in Petri dishes. Pour the each dish with 18 ml of MPA and mix gently.
- Prepare one dish only with MPA, it will be served as control of growth.
- Divide the dish from bottom to four parts by felt-tip after solidification. Innoculate bacteria in the sectors.
- Incubate dishes at 37 °C for 48 hours.
- Read the growth of bacteria (fig. 31) and write results in the table. Compare the growth of gramnegative and grampositive bacteria.

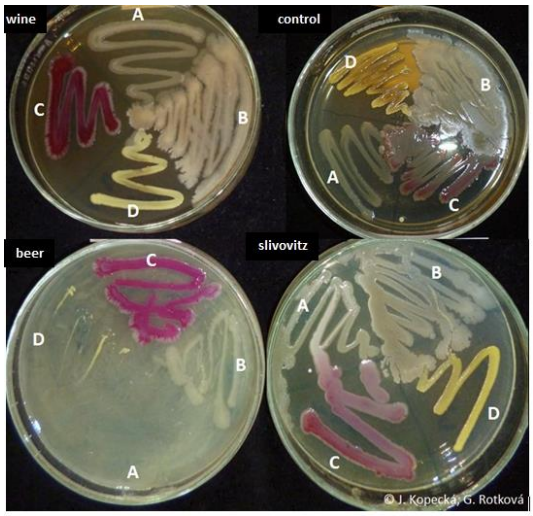


Fig. 31. Effect of alcoholic beverages on microbial growth. *Escherichia coli* CCM 3954 (A), *Bacillus subtilis* CCM 2216 (B), *Serratia marcescens* CCM 303 (C), *Micrococcus luteus* CCM 169 (D).

Conclusion

Was the inhibition of microbial growth observed? Was the inhibition of microbial growth observed only in some microbial group (grampositive, gramnegative)? Did the additional compounds in media change the character of microbial growth (appearance, pigment)?

Additional information

Fung D. Y. C., Miller R. D., Effect of dyes on bacterial growth. Appl. Microbiol., 1973, 25:793-799.

Krumwiede C., Pratt J. S., Observation on the growth of bacteria on media containing various anilin dyes. J. Exp. Med., 1914a, 19:20-27.

Krumwiede C., Pratt J. S., Further observations on the growth of bacteria on media containing various anilin dyes, with special reference to an enrichment method for typhoid and paratyphoid bacilli. J. Exp. Med., 1914b, 19:501-512.
Votava M., Kultivační půdy v lékařské mikrobiologii. Nakladatelství Hortus, Brno, 2000, ISBN 80-238-5058-X.

Control questions

1. What is the difference in sensitivity of various microbial species?
2. What provides the microbiological stability of beer?
3. What provides the microbiological stability of wine?
4. What is added into the selective media? What characteristic shlould have target compound?

Assessment of sensitivity of microorganisms to antibiotics and antibiotics concentration

Aim of practice

To determine and to compare the sensitivity of microorganicmc to the various antibiotics. To determine the concentration of unknown sample of antibiotics.

Introduction

Antibiotics (ATB) are secondary metabolites of microorganisms, are naturally synthetised to inhibit the growth of competitive organisms, provide advantages in competition about ecological niche and substrates. Among producents of ATB belong micromycetes in nature, i.e. *Penicillium, Aspergillus, Acremonium* („*Cephalosporium"), Paecilomyces*. ATB are used as chemoterapeutical agents due to their ability to affect microbial growth including pathogens. Penicillin produced by mould *Penicillium chrysogenum* (Fleming 1928) was the first described ATB, followed by streptomycin produced by actinomycete of genus *Streptomyces*. Among bacterial producents of ATB belong mostly actinomycetes: *Streptomyces griseus* (streptomycin), *S. kanamyceticus* (kanamycin), *S. erythaeus* (erythromycin), *S. venezuelae* (chloramphenicol), *S. aureofaciens* (chlortetracycline), *S. fradiae* (neomycin), *Bacillus subtilis* (bacitracin), *Paenibacillus polymyxa* (polymyxin).

Antimicrobial compounds belong together with disinfection agents among chemical methods for control of microbial growth. Among antimicrobial compounds belong antiparasitics, antimycotics, antivirotics, antituberculotics. Natural or modified ATB are used in treatment. Close-, wide-spectrum ATB or their combination are differentiated according to spectrum of effects that are administered a medicine: local, oral or by injection. Antimicrobial agents are used according to bacterial species, pH, solubility, toxicity and cost. Side effects of ATB are toxic activity (kidney, liver, placenta) and outbreak of secondary infection by damage of natural microflora. Antibiotics affect prokaryotes and do not damage function of higher organisms, damage structures only of prokaryotic cells. Important criteria are concentration of ATB, time of contact and lethal activity (bactericidal) or temporal inhibition of growth (bacteriostatic) to evaluation of antimicrobial agent effect.

Mechanisms of ATB effects

Inhibition of proteosynthesis of competitive strains: defends to initiate proteosynthesis, interferes with translation by binding on ribosome, defends to bind peptidyl tRNA on peptidyl

site (P-site), defends to elongate polypeptide on 30S or 50S subunit of ribosome, i.e. aminoglycosides, macrolides, tetracyclines, linkosamides, amphenicols.

DNA and RNA synthesis: binding of subunit DNA gyrase, defends to transcript by binding RNA polymerase, interference with bacterial DNA and RNA, bactericidal, i.e. sulphonamides, diaminopyrimidines, quinolones, rifampicin.

Inhibition of cytoplasmic membrane permeability: bactericidal, i.e. polypeptides, polyene antimycotics.

Cell wall synthesis: synthesis of peptidoglycan, affects bactericidal only growing cell by inhibition of peptidoglycan bonds formation, defends of peptidoglycan precursors movements, i.e. β-lactams, glycopeptides (penicillin, cephalosporin, vancomycin, teicoplanin, bacitracin, cycloserine).

Antagonism and competitive inhibition: synthesis of folic acid, trimetoprim, dapson, isoniazide.

Infection diseases are responsible for 40 % of all deaths all over the world considering the increasing resistance of pathogens to ATB. Resistant bacterial strains have the ability to not accept (absence of receptors, efflux systems), cleave, inactivate (i.e. β-lactamase inactivate β-lactams ATB) and secrete ATB, modify the target structures (methylation of rRNA) or enzymatic pathways. **Resistance** is **primary** that results from natural cell characteristics and functions (absent receptor, transport system, target site for ATB) and **secondary**, gained, that is caused by spontaneous genome changes by mutations or genetic information transfer by plasmid or transduction. Inappropriate using of ATB helps to secondary resistance. Resistant bacteria are present in very low frequence (occurence of mutant strains for certain characteristic is stable 10^{-8}) for short period considering to horisontal plasmids transfer by conjugation and fast reproduction of cells having plasmid (vertically = to offspings) the frequence increases fast. Spreading of resistance is increased by animal breeders and by preventative ATB treatmens of animals. Effort against spreading of bacterial resistance takes place in many areas: tightening of hygienic and epidemiological preventions, limitations of ATB usage by animals only at serious infections, surveillance of ATB using, evidence and control of nosocomial infections, informedness of doctors and patients. Antibiotics are often used unnecessarily or wrong (viral infections, noninfected diseases, irrational alteration of ATB set, early usage of ATB s.c. last generation, retreating of patients demands, early ending of ATB treatment).

Bacterial strains resistant to more ATB at same time occur today. Resistance depends on selective pressure of ATB when bacterie mobilize plasmid with genes for resistance. Function operon is changed by another and plasmid is transferred by horizontal transport among different genera.

Research of biosynthesis of new, especially hybrid ATB, takes place by genetic engeneering and combinatorial biochemistry today. Alternative to ATB is phage therapy with target effects on concrete pathogen without affecting of natural microflora.

It is very important to determinate the sensitivity of microorganism to apllied compound cosidering the application in human or veterinary medicine. Set of tests to Atb sensitivity is called antibiogram. It is essential to maintain the same time and temperature of cultivation for certain microorganism and tested compound in all microbiological methods. Sensitivity of diffusion methods depend mostly on diffusion of tested compound in agar layer, thus it is very important maintain ome conditions: constant density and humidity of agar, same thickness of agar, preparation of agar with absolute flat surface. **Mueller-Hinton agar** is used to standard test sensitivity of ATB. Medium is rich on nutrients, has lower content of solidifying compound and standard diffusion ability. Except for ATB testing, Mueller-Hinton agar is used to isolate genera *Neisseria* and *Moraxella*.

Dilution, nephelometric, titrimetric and diffusion methods are used to ATB concentration determination.

The lowest concentration of antimicrobial compound in that the bacterial growth is not yet observed is called **minimum inhibition concentration (MIC)** of antibiotic. It expresses the amount of ATB (g/ml, mg/l) that completely inhibit bacterial growth *in vitro*. MIC could be determinate by quantitative dilution method i tubes or in microtitration plate. As sensitive strain is considered strain whose MIC is 2-4x lower than concentration therapeutically reached in blood. As resistant strain is considered strain that reproduces at ATB concentration that is signifficantly higher than average MIC in the same strain.

Diffusion tests of ATB sensitivity (qualitative test) are divided according to applying of tested compounds. **Drop** methods – compounds is dropped on surface of solid medium. **Disc** methods use discs of filter papere that are saturated by tested compound. Discs are placed on solid agar (routine testing of pathogenic microorganisms sensitivity to ATB). **Well** method use wells deeped by cork-borer direct in agar layer in that the tested compounds are pipetted. **Quantitative dilution E-test** is the paper saturated by decreasing concentration of ATB. Clarifying of bacterial growth in pear-shaped zone of sensitivity to ATB and MIC value is observed.

Assessment of sensitivity to antibiotics by diffusion test – disc method

Assessment of sensitivity is mostly performed by qualitative diffusion test in agar medium. Tested strain is smeared uniformly on agar surface, paper discs saturated by ATB (commercial, saturated by defined amooount of ATB) are applied on bacterial smearing. Concentration of ATB is mentioned on every disc. Antibiotic diffuses from disc horizontally to agar in concentration gradient during cultivation. Active substance is indicated by circular **inhibition zone** formation around disc. Sensitivity of microorganism to tested compound is determinated from size of inhibition zone. Size of zone is influenced by ability of antimicrobial compound to diffuse through agar and by speed of microbial growth. Limiting point of resistance is individual for every microbial strain and target ATB. Accuracy of test is control by standard bacterial strains. Averages of inhibition zones are comparable with MIC values (semiquantitative method) at maintaining exact conditions (quality of agar, pH, ions concentration, inoculum amount).

Assessment of ATB concentration

Diffusion well method could be used to assess concentration of compound inhibing growth of bacterial strain. Unknown concentration of ATB sample is determinated from calibration curve. Values to design calibration curve are determinated from several sizes of inhibition zones measured on several dishes with defined concentration of tested ATB. Zones are formed around 4 wells in that the ATB solution with defined concentration was pipetted. Calibration curve (relationship of zone average in mm and logarithm of concentration) is constructed from average of inhibitions zones values with standard solution. Advantage of well method is not strictly sterile conditions during manipulation with tested compound, diffusion of active substance is not substantial influenced by other compounds, method is sufficiently quick and sensitive. Disadvantage of method is laborious preparation of wells and the possibility of overpouring of tested compound by manipulation with dishes.

Instruments and microorganisms

Petri dishes with Mueller-Hinton agar

Sterile cotton-wool swabs, pincette, cork-borer, scalpels

Antimicrobial discs, standards and samples of oxacillin

Automatic pipette, sterile tips, rule

ATB: erythromycin (macrolides of 1. generation), vancomycin (glycopeptides), rifampicin (ansamycines), chloramohenicol (amphenicoles), cephalothin (cephalosporines of I. generation), nitrofurantoin, oxacillin (isoxazolylpenicillines, penicillinase resistant), tetracycline (cyclines), co-trimoxazol (sulfonamides), penicillin (beta-lactams, dipeptides)

MJ (in czech: mezinárodní jednotka; IU= International Unit) is a unit of measurement for the amount of substance, the unit is based on biological aktivity or effect

Micrococcus luteus CCM 169

Staphylococcus saprophyticus subsp. saprophyticus CCM 2354

Bacillus cereus CCM 2010

Proteus vulgaris CCM 1799

Escherichia coli CCM 3954

Staphylococcus aureus NCTC 8511

Providencia rettgeri CCM 5618

Methods

Sensitivity of microorganisms to antibiotics – diffusion disc method

- Pipette 0,2 ml of liquid bacterial culture on surface of Mueller-Hinton agar and smear it by the sterile stick or by the cotton-wool swab (dense smear).
- Putt the test antibiotic discs **aseptically** on dish by sterile needle. Discs have to be putted in adequate distance from each other and from the edge of dish.
- Incubate for 24-36 hours at 37 °C.

Evaluation: Observe the microbial growth around the antibiotic discs (fig. 32). Measure the size of inhibition zones as lenght of abscissas of zone average in two vertical directions (the size of disc is included in evaluation). Calculate the arithmetic average and assess the sensitivity: insensitive microorganism (inhibition zone until 11 mm), sensitive microorganism (inhibition zone 11-17 mm), very sensitive microorganism (inhibition zone over 17 mm).

Note: Mentioned ranges of inhibition zones are only orientation for testing of microbial sensitivity. Actualised tables are used in medical practice. The tables introduce sizes of zones for target antibiotic and species. The increased resistence to antibiotics is the reason for actualisation of tables.

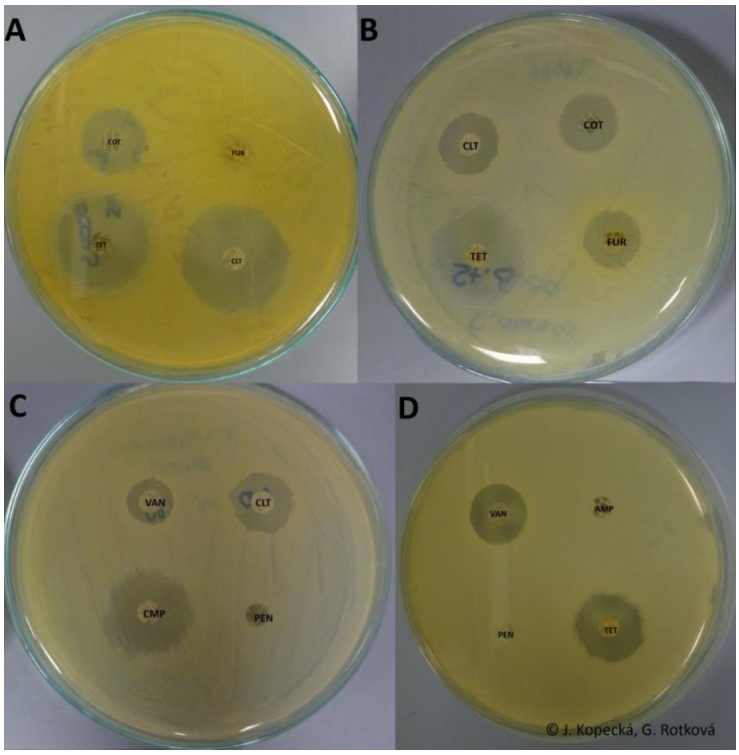


Fig. 32. Assessment of bacterial sensitivity to antibiotics by disc method, *M. luteus* (A), *S. aureus* (B, C), *B. cereus* (D); vancomycin (VAN), chloramphenicol (CMP), ampicillin (AMP), cefalotine (CLT), nitrofurantoin (FUR), tetracycline (TET), co-trimoxazole (COT), penicillin (PEN).

Assessment of oxacillin concentration by well diffusion method

- Boil the Mueller-Hinton agar (15 ml in tubes) and keep it warm at 45 °C.
- Innoculate 0,5 ml of inoculum *S. aureus* NCTC 8511 in tubes.
- Mix the volume of tubes, pour it in sterile dishes and let it solidify on straight surface.
- Prepare the standard set of oxacillin dilution, dilute in distilled water to concentration: 250; 125; 62,5; 31,25; 15,625; 7,81 μg/ml.
- Make 4 wells by cork-borer and scalpel after solidification of agar; cork-borer and scalpel sterilize by flame after plunging in ethanol. Put the extracted parts of agar in separated Petri dish (to likvidation, parts contain *S. aureus*). Sterilize cork-borer and scalpel after work.
- Sign dishes, 1 standard concentration or 1 sample is always on 1 dish.
- Pipette 40 μl of antibiotic solution into each well, solution of 1 concentration is always on 1 dish. It is necessary to transfer dishes carefully because of liquid in wells.
- Incubate for 24 hours at 37 °C.

Evaluation: Measure the averages of zones. Calculate the average valu efor every zone (from 4 zones on dish for target concentration). Construct the calibration line in MS Exel (relationship of zone average in mm to logaritmus of oxacillin concentration) from values of standard solution (fig. 33). Asses the concentration of samples (µg/ml) from values of calibration line (equation of regression). Number of sample and its concentration will be introduced in protocol.

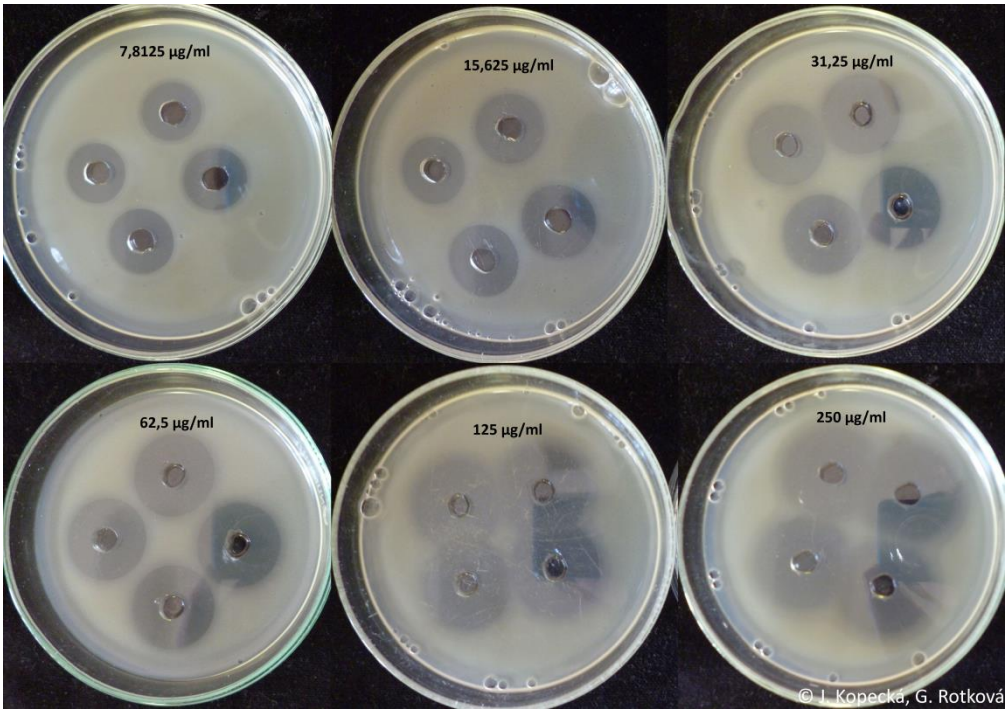


Fig. 33. Standard set of ATB dilution (oxacillin) on Mueller-Hinton agar and the size of inhibition zones - *S. aureus* NCTC 8511.

Assesment of antagonists - natural producents of antibiotics

- Innoculate in one line the probable producent (P) of antimicrobial compounds (*Streptomyces*, *B. subtilis*, *P. polymyxa* or *S. marcescens*), see fig. 34, on Mueller-Hinton agar.
- Innoculate the tested bacterial strains (TK) in lines vertically to the producent.
- Incubate for 24-36 hous at 37 °C.
- Evaluate the possible inhibition of growth of tested bacterial strains.

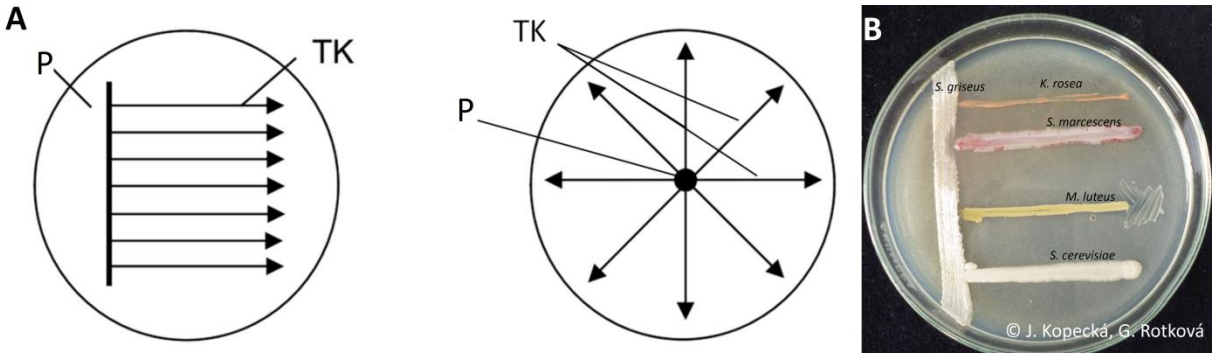


Fig. 34. Innoculation of possible producent of antimicrobial compounds (P) and tested strains (TK) of bacteria (A), real sample (B); negative effect on growth *S. griseus* is visible in *S. marcescens*.

Conclusion

Compare the sensitivity of microorganisms to various ATB. Is the sensitivity of various species to one antibiotic the same? Is the effect of one antibiotic same to various microorganisms? Did you succeed in drawing up the calibration curve and in determination of concentration ofunknown antibiotic sample?

Additional information

Greenwood D., Slack R. C. B., Peuthere a kol., Lékařská mikrobiologie. GRADA Publishing, 1999, ISBN 80-7169-365-0.

Votava M., Kultivační půdy v lékařské mikrobiologii. Nakladatelství Hortus, Brno, 2000, ISBN 80-238-5058-X.

Určování citlivosti a rezistence bakteriální kultury k antibakteriálním látkám (http://fvl2.vfu.cz/sekce_ustavy/mikrobiologie/obrazova_priloha/mikrob/6.html, 2. 3. 2016)

Nová látka klíčem ke stovkám antibiotik (http://www.gate2biotech.cz/nova-latka-klicem-ke-stovkam-antibiotik/, 2. 3. 2016).

Nouza K., Nouza M., Antibiotika – hrozí konec éry? Medicína 3, VI, 1999. (http://www.zdrava-rodina.cz/med/med399/med399_42.html, 17. 7. 2016).

Eropean Antimicrobial Resistance Surveillance Network (http://ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx, 3. 3. 2016).

Control questions

1. What are the mechanisms of bacterial resistance to antibiotics?

2. Is the antibiotic produc of primary metabolism? For what are antibiotics beneficial in environment?
3. Describe the term inhibition zone.
4. What is the difference between antibiotic and chemoterapeutic?
5. For what is used disc diffusion method and what is its procedure?
6. Write the name of medium that is used for testing of sensitivity to ATB?
7. Why is dilution set of antibiotic prepared during the estimation of ATB concentration?
8. What is the MIC?

Bacteriocins

Aim of practice

Proof of production of antibacterial compounds – bacteriocins.

Introduction

Bacteriocins present the group of antimicrobial proteins characterised by specific spectrum of efficiency. Production of bacteriocins prefers bacteria in natural environment, facilitates elimination of other bacteria of the same or sibling species in competition of energy sources. Nowadays, 3 basic group of bacteriocins are defined - colicins, microcins and corpuscular bacteriocins.

Colicins are high molecular proteins (25-80 kDa) produced by *Escherichia coli* and other genera of family *Enterobacteriace*. Microcins are low molecular compounds (to 10 kDa) of peptides character produced by strains of family *Enterobacteriaceae*. Todays, 25 types of colicins and 12 types of microcins is well characterised. Microcins differ by the efficiency spectrum, higher stability to extremely pH, temperature, higher resistence to proteases from colicins. Colicins are coded on plasmids, while microcins could be coded on plasmid or on chromosome.Corpuscular bacteriocins are high molecular particles similar to phage flagellum (phage tail-like bacteriocins). Occurence of corpuscular bacteriocins was observed in species *Budvicia aquatica, Pseudomonas aeruginosa* and *Pragia fontium*.

It is expected that bacteriocins produced by *E. coli* could be employed as probiotics and antibiotics. Nonpathogenic strain *E. coli* Nissle 1917, producent of microcins H47 and M, is

used very often as probiotics. Strain was isolated by german bacteriologist Alfred Nissle during the first world war and was used as prevention of soldiers to infection diarrhoea. Presently, strain is the activne substance of Mutaflor® probiotics.

Usage of probiotics leads to activation of organism immunity. Effect of strain Nissle 1917 and also effect of microcins H47 and M production was tested on AIEC (adherent-invasive *E. coli*) strains isolanted from biopsy of patients with Crohn disease. This strain decreases significantly the ability of AIEC strains to adhere and to invade in epithel and is recommended as therapy during chronic intestinal diseases.

Instruments and microorganisms

Escherichia coli – human intestinal isolates

Indicator strain – *E. coli* K12 – ROW

1,2 % TY agar (8 g enzymatic lysate of casein, 5 g yeast extract, 5 g NaCl, 1 l H_2O, 12 g agar)

NA agar (28 g agar, 1 l H_2O)

Chloroform

Methods

- Innoculate strains of *E. coli* by the stabbing in agar (12 stabs/strains on dish).
- It can be used the basic medium 1,2 % TY agar, 1,2 % TY agar with mitomycin C (indication of SOS answear, increased production of some bacteriocins types), 1,2 % TY agar with trypsin (decomposition of bacteriocins with protein character) or NA agar (medium with low nutrient amount for incresing of production of some bacteriocins types).
- Cultivation is performed for 48 hours at 37 °C.
- Kill strains by adding of chloroform for release of bacteriocins in medium. Place the cellulose on the lid of dish, pipette 1 ml of chloroform and let it work for 30 minutes.
- Add 100 µl of indicator culture in 3 ml of 0,66 % TY agar (agar is temperated at 45 °C).
- Pipette 3 ml of 0,66 % TY agar temperated at 45 °C on medium with killed strains and let it solidify.
- Cultivation is performed for 24 hours at 37 °C.
- Strains producing bacteriocins form inhibition zones (fig. 35).

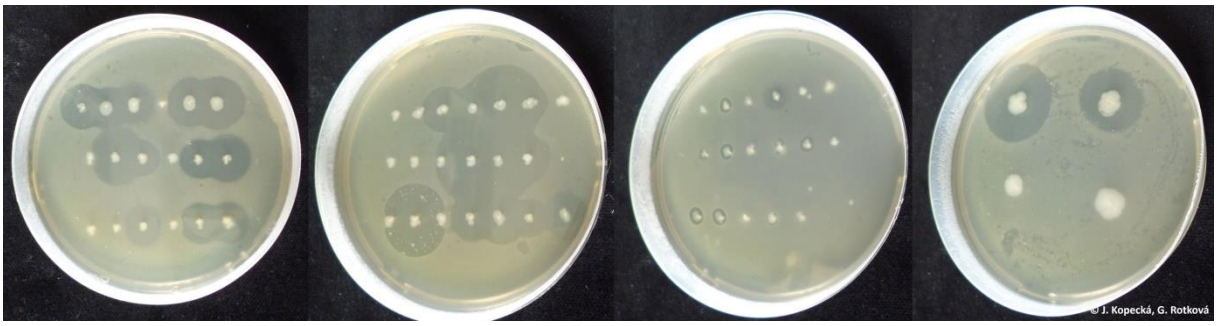


Fig. 35. Formation of inhibition zones by *E. coli* producing bacteriocins, to which is sensitive indicator strain.

Conclusion

Did all strain produce bacteriocins (formation of inhibition zones)? Why is necessary to kill producing bacteria?

Additional information

Micenková L., Bactericinogeny in pathogenic and commensal *Escharichia coli* strains. Disertation thesis, MU Brno, 2016.

Micenková L., Molekulárna typizácia bakteriocínov z ľudských kmeňov *E. coli*. Diploma thesis, MU Brno, 2011.

Control questions

1. What is the requirement of suitable indicator strain?
2. What types of bacteriocins do you know?
3. What is the signifikance of bacteriocins for intestinal bacteria?

Evidence and isolation of target soil microorganisms

Aim of practice

Isolation and proof of 3 groups of microorganisms (*Azotobacter*, *Clostridium*, cellulolytic bacteria) from the soil sample due to the selective condition of cultivation.

Introduction

Characteristic of soil sample (soil fertility, amount of humus, texture, amount of oxygen, acidity, profile, depth) is considered by qualitative or quantitative evaluation of microorganism species representation. The most of microorganisms live up to depth of 10 cm. Several trillions of cells are present in 1 g of soil. We could prove them by *in situ* detection (fluorescence, nucleid sonds) or by cultivation according to their characteristic metabolic activities: nitrogen fixation, sulphur oxidation, sulphates reduction, urea and cellulose decomposition. Preparation from soil extract could show information about morphology and stainability of soil cells.

Bacterial genera *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Nocardia*, *Pseudomonas*, *Serratia*, *Streptomyces* including moulds (*Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*) belong among common soil microflora. Some of them could be human, animals and plants pathogens (*Actinomyces*, *Clostridium*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Streptomyces*). Microorganisms occur in pure cultures very rarely in nature, they form microbial community. High number of microbial species ocurs in normal conditions (neutral pH, sufficient amount of nutrients and water) in environment. Certain bacterial group outweights in soil of certain character, i.e. waterlogged soil supports growth of anaerobes, thermophilic bacteria occurs mostly in compost, moulds are mostly in acidic soils. Lower number of species and higher number of one resistant species is typical for extreme conditions. Dominant species occurs in population with higher number of CFU/g than other species. Microbial community is opened and dynamic system.

Winogradsky defined two basic bacterial groups according to culminating number of representatives depending on nutrient sources. A**utochthonous bacteria** – natural, typical organisms that are represented in relativelly high and constant number for the whole year independently on nutrient amount. Low metabolic activity is typical for them. They are clasified accoding to cell morphology (stained preparation), i.e. actinomycetes,

Agrobacterium, Streptomyces, Nocardia. Presence of **zymogenic (alochthonous) bacteria** depends on actual increased concentration of nutrients, addition of specific compounds that are quickly utilised. They have high metabolic activity nd participate significantly on soil mineralization, elements circulation in biosphere, i.e. nitrifying bacteria, cellulolytic bacteria, sulphur oxidation, myxobacteria, *Bacillus, Flavobacterium, Mycobacterium, Pseudomonas.*

Microorganisms participate on soil conversion and modify environment by their activity. Energy enters in soil ecosystem in form of sun light or organic and inorganic compounds. Organic compounds are oxidated to CO_2 by respiration or are fermented to reduced compounds. Chemolitothrophic organisms oxidate inorganic compounds and participate to synthesis of organic compounds by autotrophic activities. In general, it is mineralization process (conversion of organic binded element to inorganic form), imobilization (conversion of inorganic elements to organic complexes), oxidation, reduction, fixation or volatilization (conversion of gas form to another), processes of decompozition (humification, mineralization), elements circulation, oxidation of toxic nitrites to nitrates (nitrifying bacteria), oxidation of sulphides to sulphates (*Thiobacillus*), bioremediation, decompositin of highly decomposed compounds (i.e. pesticides, cellulose, chitin), production of compounds affecting growth of plants, nitrogen fixation by symbiotic (*Rhizobium*) and free living (asociative symbiotic *Agrobacterium, Azotobacter, Clostridium*) bacteria, production of secondary metabolites (bacteriocins, antibiotics), supression of moulds disease of plants (*Bacillus megaterium, B. subtilis, Pseudomonas fluorescens*).

Microorganisms have to be isolated and cultivated in pure cultures to work only with specific bacterial group. Selective media could be used to isolation. Medium containing no nitrogen source could be used for isolation of nitrogen fixating bacteria. Some photothrophic, chemothrophic bacteria (symbiotic and free living) and cyanobacteria (*Azotobacter, Klebsiella, Rhizobium, Clostridium pasteurianum, Rhodospirillum, Anabena, Nostoc*) have enzyme nitrogenase that is able to fix nitrogen from air. It is energetically demanding process (consumption of 15 ATP/ 1 molecule of N_2).

Cellulose utilization could be prooved by characteristic growth on materials containing cellulose as source of carbon. Marker of soil fertility is the presence of **cellulolytic bacteria**. Fast growing species of genera *Cytophaga, Cellvibrio, Cellfalcicula, Sporocytophaga* occur in intensively cultivated soils; myxobacteria occur in moderately cultivated soils; moulds occur mostly in weak cultivated and acidic soils. The most common and water-insoluble polysaccharide cellulose is the base of plant cell walls together with hemicelluloses, pectins, lignin and fats. Cellulose is utilised aerobically and could be fermented anaerobically also.

Cellulose is hydrolytically cleaved by exoenzyme cellulase to cellobiose outside the cell that is cleaved by endoenzyme cellobiase to two subunits of glucose after transport inside the cell. The speed of cellulose decomposition is affected by amount of cellulolytic bacteria and by the presence of certain compounds, cellulose is cleaved more slowly in presence of lignin.

Characteristic of genus *Clostridium:* Pleomorphic grampositive rods, single, pairs or in short chains. Peritrichous, oval or round endospores formation. Obligatory anaerobic, some strains aerotolerant. Temperature optimum of growth 10 – 65 °C. Some strains fix gas nitrogen and form toxins. Occurence in soil, muds, sea sediments, rests of plants, gastrointestinal tract of animals and human, clinic material. Identification according to proteins and saccharides utilisation - saccharolytic, proteolytic and both saccharolytic and proteolytic. Primary and opportune pathogens.

C. pasteurianum - strictly anaerobic, butyric fermentation, tolerant to acidic and soaked soil, lower temperatures; spores formation, occurence in all types of soil (10^3 - 10^5 CFU/g).

Characteristic of genus *Azotobacter*: Gramnegative ovoid pleomorphic rods or cocci, single, pairs or irregular clusters. Peritrichous, aerobic, chemoorganothropic. Cysts and pigments formation. Nonsymbiotic nitrogen fixation or asociative symbiotically (*Azotobacter paspali* with grass *Paspalum notatum*). Cultivation on medium with molybdenum or vanadium. Occurence in soil or water.

A. chroococcum – occurence in lower number (10^2-10^4 CFU/g) in aerated soil with neutral or weakly alkaline pH near to root system of plants. It does not fix nitrogen in acidic soil. It demands presence of sugars, simple alcohols, phosphorus, calcium, molybdenum, boron, vanadium, iron and manganese. Temperature optimum at 25 - 30 °C.

Instruments and microorganisms

Petri dishes with Ashby agar, empty plastic Petri dishes

MPB with 5% of glucose

Tubes, pipettes, loops, gas burner, water bath

Sterile paraffin oil

Filter paper, parts of journal or newspapers, cellulose

Soil (greenhouse, soil from wood or forrest, garden, sand pit, plant pot, compost)

Methods

Isolation of genus *Azotobacter*

- Innoculate Petri dishes with Ashby agar (selective nitrogen-free medium eliminates species demanding presence of nitrogen, it does not eliminate of micromycetes) direct by the parts of soil.
- Cultivation is performed for 72 hours at 25 – 30 °C, do not turn the dishes.

Evaluation: Growth of genus *Azotobacter* shows mucilaginous colonies around the parts of soil (fig. 36). Colonies areecome brown. The characteristic smell of soil is smelled. Gramnegative rods in pairs could be observed by microscopic control in preparation. Cells are surrounded by capsules that could be highlighted by negative staining. Static stadia, cysts, could be observed in preparation.

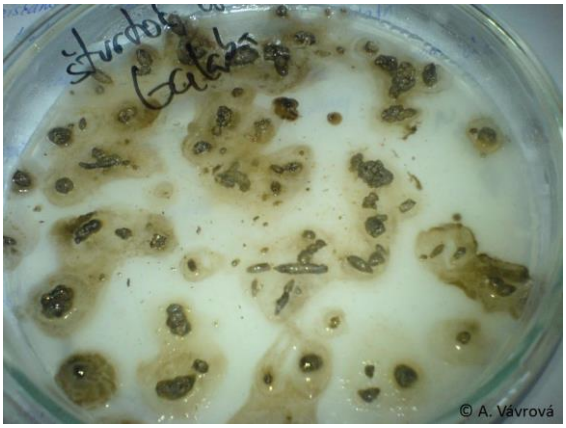


Fig. 36. Isolation of genus *Azotobacter* on Ashby agar.

Isolace of spores of genus *Clostridium*

- Prepare the soil extract by mixing of 10 g of soil and 100 ml of distilled water. Mix the solution for 10 minutes and filtre the upper part through double filter paper.
- Pour the suspension (cca 2 ml) in tube. Place it in water bath (75-80 °C) for 15 minutes (pasteurization, killing of vegetative cells, spores survive).
- Pipette 1 ml of pasteurized soil extract in warm sterile medium (MPB with 5 % of glucose). Pipette immediately 1 ml of sterile paraffin on the medium for establishing anaerobic environment.

Evaluation: The sediment, gas and characteristic smell of butyric fermentation is originated in presence of clostridia (fig. 37). Grampositive rods, alternatively spores, could be observed in preparation (phase contrast/brief filed for Gram staining) by microscopic control.

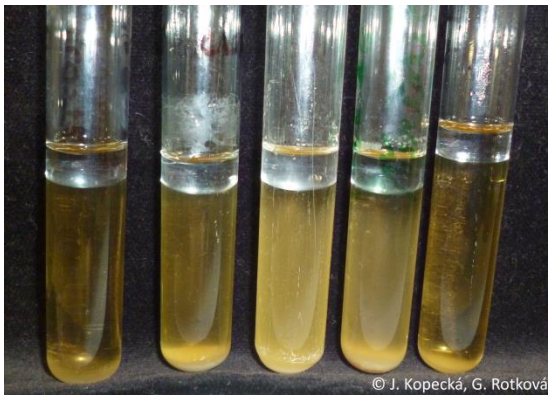


Fig. 37. Presence of sediment after anaerobic spores of genus *Clostridium* germination.

Evidence of cellulolytic bacteria

- Spread the soil in Petri dishes in height circa 2/3 of dish and moisturize it properly.
- Place the single stripes of filter paper, cellulose and newspaper by the pincette (1 cm gap) and moisturize again.
- Incubate at room temperature, moisturize the soil continuously. Incubation is performed for 3 weeks, evaluation is performed after 7 days (fig. 38).

Evaluation: Observe and record possible decomposition and changes in pigmentation (presence of varoious colours) of various sources of cellulose. The most effective decomposition should be in cellulose (cca 50 %), lower at filter papaer (30 %) and the lowest decomposition shloud be at newspaper due to the presence of printing dye. The most cellulolytic bacteria contains soil with higher amount of humusu, lower numbers of bacteria are in sand soils.

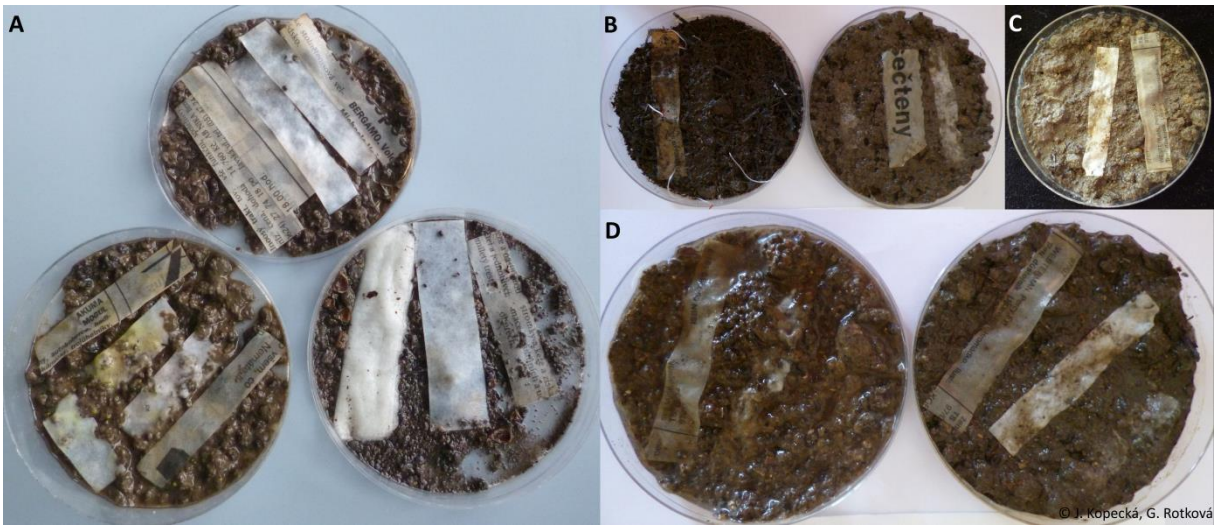


Fig. 38. Decomposition of different sources of cellulose by cellulolytic microorganisms after one (A) and three (B, C, D) weeks of incubation.

Conclusion

Additional information

Němec M., Mazal P., Cvičení z mikrobiologie, Učební text VŠ. 1989, Brno.

Möllerová J., Symbiotická fixace dusíku, Živa, 2006 (http://ziva.avcr.cz/files/ziva/pdf/ symbioticka -fixace-dusiku-bakterie-rhizobium-s-l-a.pdf, 7. 3. 2016).

Enzym nitrogenáza a chemizmus dusíku (http://chem.rochester.edu/~plhgrp/ nitrfix.html, 7. 3. 2016).

Control questions

1. What is the principle of isolation target bacterial groups?
2. Why only the spores germinate in the proces of isolation of genus *Clostridium*?
3. In what environment (in the term of oxygen) germinate spores of genus *Clostridium*? How are the spores isolated and what characteristics do we observe in the case of positive isolation?
4. Write some function of soil microorganisms.
5. Write some bacterial genera commonly living in soil.
6. What selective medium is used for *Azotobacter* isolation, what is the principle of isolation?
7. Do bacteria need symbiosis with plants for nitrogen fixation?
8. Write the name of enzyme which is needed for bacterial air nitrogen fixation? Does the enzyme work aerobically on the dish?
9. Describe the term autochthonnous/alochthonnous.

Winogradsky column

Aim of practice

Create the Winogradsky column and observe the separation of individual layers in time.

Introduction

Sergei Nikolaievich Winogradsky, russian microbiologist, attended to soil microbiology mostly. He discovered iron, sulphur and ammonia oxidating bacteria that are capable to bind CO_2 in organic material. He isolated anaerobic bacteria that fix nitrogen and he studied decomposition of cellulose. **Winogradsky column** (fig. 39) is the form of microcosmus in that microorganisms and nutrients interact in vertical gradient. Column demostrates various functions of microorganisms in nature, when activity of one organism facilitates the growth of other. Column is complex, self.sufficient and recycle system that is supplied only by light energy. Products of fermentation and sulphides rise from reduced zones, oxygen penetrates from surface in column. Layers like in nutrient rich sediments are formed. Photosynthetising organisms obtain energy from light.

Mud is mixed with sodium sulphate, sodium carbonate and torned newspapers (source of cellulose). Water is added in column, incubation is performed on light. Series of reaction starts in column, specific microorganisms create special microclima as the answear to chemical gradients.

Cellulose is degraded on the bottom of column (genus *Clostridium*). Products of fermentation are available for another microorganisms as reductants, sulphate is utilised as oxidant. Genus *Desulfovibrio* produces hydrogen sulphide that rises up to oxygenic zone and creates stabile gradient of hydrogen sulphide in that phototropic *Chlorobium* and *Chromatium* create visible green and purple zone. These microorganisms utilise hydrogen sulphide as source of electrons and CO_2 from sodium carbonate as source of carbon. Purple nonsulphur bacteria of genus *Rhodospirillum* and *Rhodopseudomonas* could grow over this layer. These photoheterotrophs utilise organic material as donors of electrons in anaerobic conditions. Oxygen and hydrogen sulphide could be present in higher layers in column, where are utilised by adapted microorganisms (*Beggiatoa*, *Thiothrix*) that utilise reduced sulphur compounds. Alga (diatom) and cyanobacteria could be visible in upper layer of column.

Oxygen and CO_2 create concentration gradient in column. Oxygen is dissolved in water only in limited amount, solving of oxygen depends on temperature of water, pressure and dissolved

salts. Temperature and pressure affect significantly the amount of oxygen that is available for microorganisms. Concentration of oxygen could be significantly higher at lower temperatures. Quick decline of dissolved oxygen in water could occur during water contamination by nutrients that leads mostly to killing of fishes. CO_2 participates on many chemical and biological processes, i.e. affects pH of water. If autotrophic microorganisms (i.e. alga) utilise CO_2, pH of water increases.

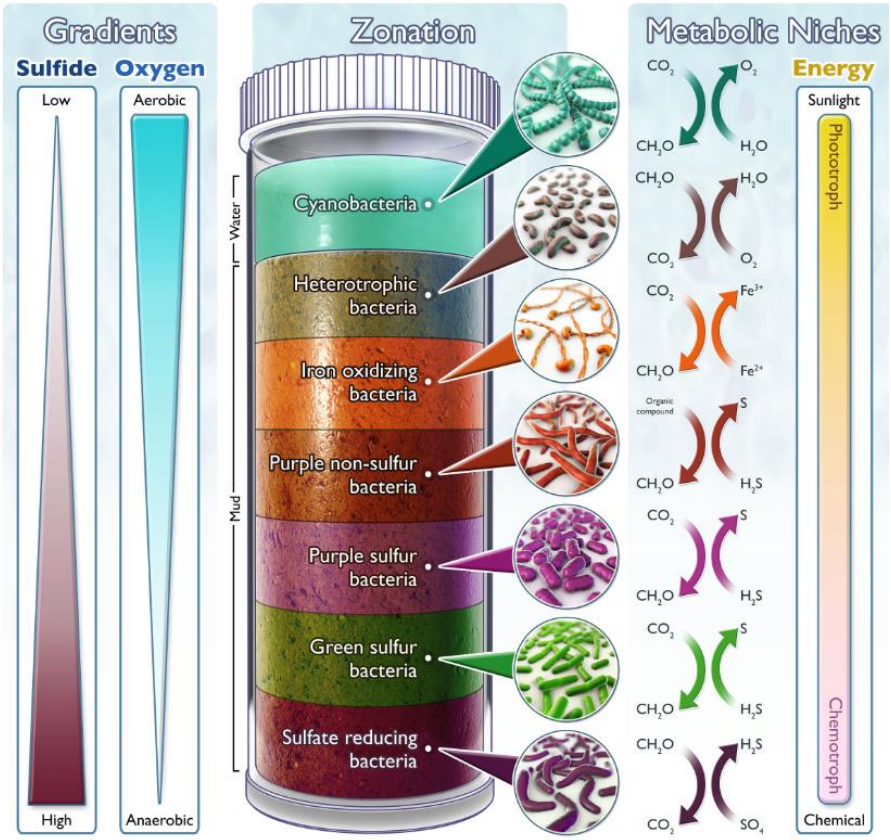


Fig. 39. Winogradsky column, microbial evolution in the bottle (http://www.hhmi.org /biointeractive/poster-winogradsky-column-microbial-evolution-bottle, 8. 3. 2016).

Instruments and microorganisms

Mud and water from river

Egg, filter paper

Methods

- Mix mud with egg (Na_2S and $CaCO_3$), torned filter paper (cellulase) and water.
- Fill the cylinder by the mixture, add water and close the cylinder.
- Incubation is performed on light at room temperature. Separation of individual layer can be observed after time (fig. 40).

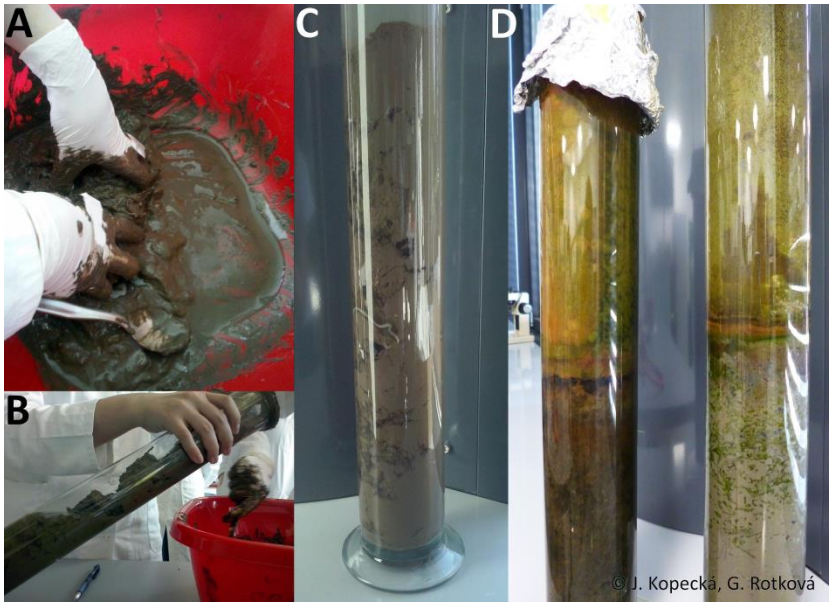


Fig. 40. Preparation of Winogradsky column (A, B), start of incubation (C) and separation of layers after a few months incubation (D).

Conclusion

Did the layers separate? Were the individual layers good observable? After what time did the layers separate?

Additional information

Prescott L., M., Harley J. P., Klein D. A., Microbiology, WCB, Dubuque, 1996, ISBN 0-697-29390-4.

Control questions

1. Why is the egg added into the column?
2. Filter paper serves as source of what in the column?
3. Is it necessary to add some nutrients into the column during the incubation?
4. Which layers could we observe in the column?

Observation of bacterial spores and their staining, negative staining

Aim of practice

Highlighting of cell – genus *Bacillus* by negative staining (nonfixed preparation). Observing of bacterial endospores of genus *Bacillus* in native preparation (phase contrast). Highlighting of capsules - genus *Azotobacter* by negative staining. Spores staining.

Introduction

Besides growing and dividing vegetative cells, the structure for long-term surviving of unfavourable conditions are present in prokaryotes – **endospores**. Endospores are static resistant stadia. Only one single endospore is present in prokayotic cell. Spore contains peptidoglycan that has completely different character from cell wall peptidoglycan. Macromolecules are stabilized by presence of specific proteins, decreasing of water amount, increasing of calcium content in spore. Calcium is binded in dipicolinic acid that is presented only inside of bacterial endospores in nature, it provides thermoresistance to spore. Spores are resistant against to UV and γ radiation, drying, lysosyme, changes of temperature, lack of nutrients, many disinfection agents treatment due to the presence of many coats of various character. Bacteria could be spreadded to very long distances in various environments due to spores. Spore formatin is not the answear to anfavourable conditions, but it is the preparation on it. Spores are highly refractive objects. Endospores are nonreproducing structures of small number of mostly grampositive bacteria (i.e. *Bacillus*, *Clostridum*, *Thermoactinomyces*, *Desulfotomaculum*, *Sporosarcina*, *Sporolactobacillus*, *Oscillospira*) and gramnegative bacteria (*Coxiella burnetii*).

Thermostable spores are significant clinically, pharmaceutically and technologically, especially genera *Bacillus* and *Clostridium*. Bacterial spores are important agent of bioterorism (i.e. *Bacillus anthracis* - anthrax). Some spores are used as biopesticides (i.e. Bt toxin, spore protein from *Bacillus thuringiensis* var. *israelensis*). Spores contain proteins, peptides or enzymes on their surface that are used as specific probes or have biocatalytic function. Modificated spores of *B. subtilis* are used as vehicle of vaccine and another pharmaceuticals, i.e. surface proteins of spore *B. subtilis* containing fragment C of tetanus toxinu; alfa toxin *Clostridium perfrigens* used for oral and nasal immunization of human and animals. Spores as pharmaceutical vehicles provide thermal stability, flexibility for genetic modifications and inexpensive process of production.

Whereas toxins of sporulating species are mostly thermolabile and are inactivated after 5 minutes at 60 °C treatment, spores are very resistant. Sporulating cells resist at 100 °C for 90 minutes in species *Clostridium botulinum*, nonsporulating cells die after 30 minutes at 70 °C. Spores of *Clostridium tetani* (pathogen - tetanus) are inactivated after 20 minutes at 121 °C at 0,2 Mpa and after 90 – 180 minutes at 160 - 200 °C of dry heat. Spores are highly thermoresistant, they survive ever 5-hours boiling. Spores of *Geobacillus stearothermophilus* are used to verification of sterilizationbecause spore survive at 120 °C for 12 minutes.
Sporicidal compounds is very low amount and are expensive, i.e. ethylenoxide, β-propionlactone, concentrated bases and acids, formaldehyde with long-term exposition, peracetic acid, iodine preparates, chloramine.

Construction of matured bacterial spore (fig. 41)
Core of spore creates sporoplast (protoplast). Stroma of spore is gel matrix composed from bacterial nuclear equivalent (nukleoid), ribosomes, calcium-dipicolinate (up 10 % of dry maas) or pyridin-2,6-dicarboxyl acid replacing water during reteining of quarter structure, SASPs (small acid-soluble proteins) that are tight binded to nucleid acid and are responsible for its condensation and resistance against to UV radiation and DNA-destroying compounds. Polyamines, aminoacids and 3-phosphoglycerate are presented. Specific compounds in crystal and toxin form are presented in core of spore in some species. Core is coated by internal lipoprotein membrane, intina, that defends of chemical compound permeation from environment. Rest of original cell wall serves as the base for new cell wall during germination of endospore. Cortex composing from internal (20 %) and external part (80 %) is responsible for inpermeability and thermoresistance after core dehydratation and composes from peptidoglycanes. Circa 20-30 % of peptidoglycan units is correspondent with cell wall peptidoglycan units, rest of 50-60 % units comprise N-acetylmuramic acid modificated in N-acetylmuramyl–lactam, another 18-20 % of N-acetylmuramic acid is binded with L-alanine instead of tetrapeptide. Modifications are established by membrane binded enzymes. Pericortical external lipoprotein membrane of coat, extina, is composed from proteins rich on cystein. Extina establishes resistance to chemicals treatment. Exosporium is not present in every taxons, is responsible for resistance to chemical compounds and enzymes.

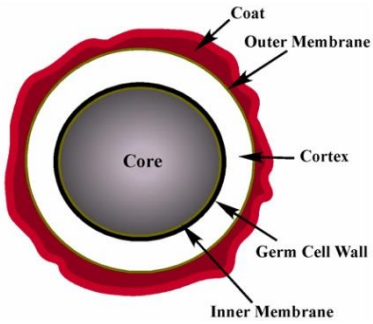


Fig. 41. Structure of bacterial endospore (https://micro.cornell.edu/research /epulopiscium/ bacterial-endospores, 8. 3. 2016).

Shape and location of spore (fig. 42) in cell is important characteristic identification marker. Oval spores are always present in i.e. species *Bacillus anthracis, B. cereus, Clostridium botulinum*; species *Clostridium tetani, Bacillus sphaericus* have round spores. Bulge caused by spore is evaluated too. Cell location could be terminal (end of rod, i.e. *C. tetani* – drum-stick, *Geobacillus stearothermophilus*), central (*C. histolyticum, C. novyi, C. septicum, B. anthracis, B. cereus*) or mostly subterminal ort paracentral (between middle on pole of cell, i.e. *C. botulinum, C. sporogenes, B. brevis*).

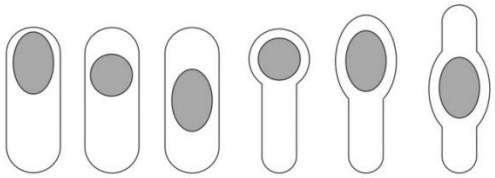


Fig. 42. Possible location of endospore and potential bulge as identification marker (http://ttktamop.elte.hu/online-tananyagok/practical_microbiology/ch06s04.html, 8. 3. 2016, adjusted).

Nonstained endospores could be observed by **phase** (shining spores; after cortex formation) and **Nomarski contrast** (plastic surface of cell, only bulging spores are visible), **simple staining** (only bulging spores are visible). Direct **staining of endospore** is possible when prospore is formed (cortex formation). Prospore is permeable for dyes. Spores absorb dye improperly even after preparation fixation due to rigid cortex. They could be stained during boiling by concentrated dyes or disinfectants (as i.e. acid-fast bacteria). Examples of spore staining is method according to Wirtz-Conklin and Schaeffer-Fulton. Stained spores are decolourized by acids and another compounds (alcohols) only with difficulties. Stainability of spores depends on developmental stadium of cell, age, quality of medium, individual characteristics of microorganisms. Stainability of spores is improved by using of sporulating

media with manganese. **Native preparation** observed by phase or Nomarski contrast enables to observe real shape and cell structure undamaged by fixation and staining, is used to observe refractive cell formation that are stained only with difficulties (i.e. spores). **Phase contrast** exploits different refraction of particles in observed object. Various cell structures have different refraction indexes, image is result of compostion of images with shifted phase and waves deviated from preparation. Dark spores appear to be shiny object in preparation.

Sporulation - the process of endospore formation

Genus *Bacillus*, specially species *B. subtillis,* is used for sporulation studies. Sporulation takes place at sufficient amount of nutrient mostly in stationary phase of growth. Several phases (fig. 43) could be differentiated during sporulation of *B. subtillis*. Phases could be characterized by morphology and on molecular-biological level. Circa 50 genes coresponds to endospore formation, the process lasts in average 6 – 7 hours. It is clear during septum formation if new vegetative cell or spore originate. In the case of sporulation, cell is switching from binary division to **asymmetric division**.

Prospore is made in s.c. sporogenous zone. Genes for spore preparation are primary transcribed. Amount of volutin, enzymes is increased. Cell utilize higher amount of acetate, number of enzymes for Krebs cycle and hydrolysis is increased. Amylases, proteases, phosphatases, Dnases facilitate in process of sporulation. Axial filaments serve to unpack nucleoid in long filament and to replicate. Septum is created. It divide cell to two unequal parts, replication of cell genetic material is terminated. Genetic material goes to the cell poles. DNA of spore is no longer active. Sporogenous zone is homogenised and inflated, has another density than rest od cell. Cytoplasmic membrane proliferate around both cell parts. Prespore is coated by two membranes, intina and extina, by infolding of cytoplasmic membrane. High amount of calcium is packed in spore, dipicolinic acid is synthetized, prospora is formed. Amount of volutin is decreased. Prospora is not refractive, does not shine by phase contrast microscopy. Process of sporulation is now irreversible. Cortex formation with different composition of peptidoglycan is regulated by chromosome of mother cell. Amount of volutin is minimum during cortex formation. Dipicolinic acid is synthetizes by mother cell, its content is regulated. Calcium dipicolinate is characteristic compound presented only in bacterial endospores. Endospore is refracive, cortex is impermeable for dyes. Synthesis of doublelayered coat is running. Exosporium composed from 10 proteins, polysaccharides and lipides is formed in strains of genus *Bacillus*. Unique character of coat proteins is assessed by

chemotaxonomical methods. Endospore maturates, mother cell lyses and maturated spore is released. External architecture, number and shape of coats is species specific.

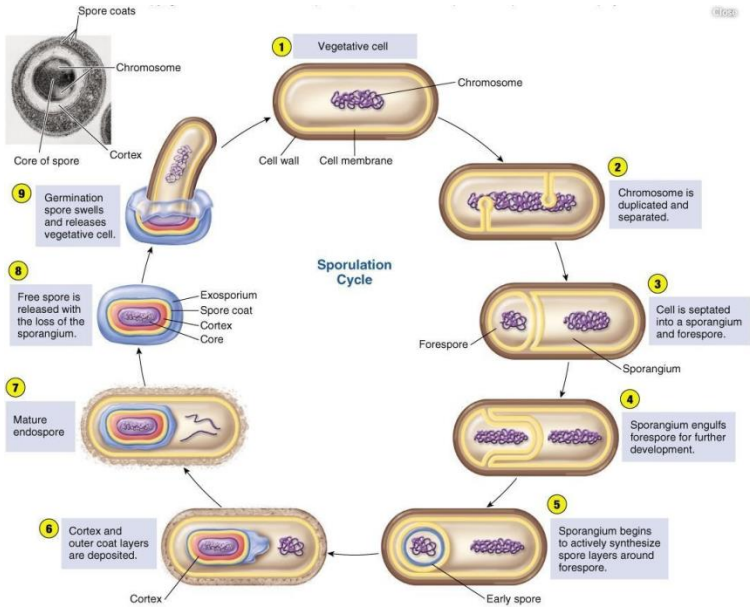


Fig. 43. Process of sporulation (https://www.quora.com/What-is-microbial-spore, 8. 3. 2016).

Germination of bacterial spores

Germination (fig. 44) starts by spontaneous activation of spore. Coat is destabilised by treatment at 70-85 °C after 5 – 10 minutes in laboratory conditions, another activators are small organic molecules. Activated spore recieves water and loses resistance. Protein stabilisators start to decompose inner parts. Aminoacids serve to form new proteins, proteosynthesis (degradation enzymes) is affected at first. When cell produces energy, regulation apparatus of chromosome starts to work (ATP = signal of chromosome activation). The first function enzymes are glycosidases to metabolise cortex, subsequently extina (phospholipids, proteins, polysaccharides). Lytic enzyme, corticohydrolase depolymerises cortex for water permeability. Division of vegetative cell is performed after 2 hours after spore germination. Terminal (on cell poles) and central germination is differentiated according to localisation of spore germination. Germination could be inhibited by presence of aminoacid D-Ala, $MgCl_2$ and by proteases inhibitor, phenylmethylsulfonylfluoride.

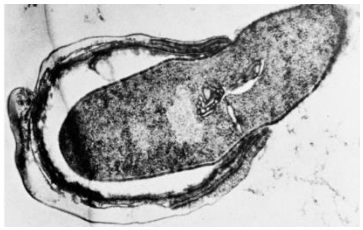


Fig. 44. Spore germination - *Clostridium pectinovorum* (Prescott et al., 1996).

Cyst of genus *Azotobacter*

Cysts are static stadia surrounded by coat (fig. 45) that protects them against to chemical and physical stress (i.e. drying, radiation). Cysts of genus *Azotobacter* contain alcylresorcinol, alginate, polyhydroxybutyrate and polysaccharides. Cyst is composd from oval cell, central body with doublelayered capsule. Cysts are more resistant to unfavouable conditions than vegetative cells, but less resistant than spores.

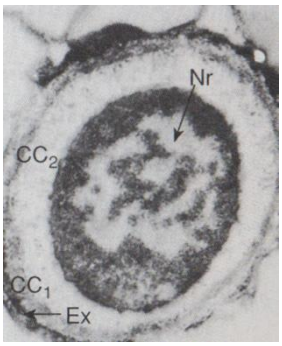


Fig. 45. Cyst structure of genus *Azotobacter*, nuclear region (Nr), outer coats (CC_1, CC_2), exosporium (Ex) (Prescott et al., 1996, adjusted).

Bacterial capsules

Polysaccharides or proteins capsules coat bacterial cell and defend it against to drying, toxins, unfavourable factors in environment. It masks bacteria against to immune answear of macroorganism in animal or human body (masking of antigen, higher resistance, factor of virulence). Bacteria forming capsules produce high amount of characteristic mucus. Capsule is clearly separated from environment compared to mucus that is more surrounded around cell. High amount of saccharides in medium or environment increases the capsules formation. Capability to form capsules could be lose by mutation.

Capsules are restrictedly stained, they could be stained after boiling in dye. Negative staining is used for highlighting of capsules – capsules are colourless, background is dark.

Negative staining

Only background of cells is stained by negative staining, no cell is stained. Size and shape is not deformed by fixation or staining. It serves for exact size measurement, determination of real shape, capsules and mucous.

Instruments and microorganisms

Microscopic slide, cover glass, innoculation loop

filter paper, sterile distilled water

nigrosin, Kongo red, methylene blue, malachite green

Bacillus megaterium CCM 2007 – round spores, no bulge, subterminal location

Bacillus sphaericus CCM 1615 – round spores, bulge, terminal location

Bacillus cereus CCM 2010 – oval spores, no bulge, central location

Bacillus thuringiensis CCM 19 – oval spores, no bulge, subterminal location

Bacillus subtilis CCM 2216 – oval spores, no bulge

Paenibacillus polymyxa CCM 1459 – oval spores, bulge, central location

Azotobacter vinelandii CCM 289 (or soil isolate)

Methods

Spores (shape, size, location) are highlighted in preparation by phase contrast.

Culture of specific age on medium supporting growth of spores is suitable for spores observing, i.e. cultures 2-3 days old are suitable for genus *Bacillus*.

Negative staining by nigrosin

- Transfer cells aseptically in the small drop of distilled water, pipette the small drop of nigrosin beside.
- Mix the drops by loop, smear it gently by another microcopic glass (applied under angle of 45˚) over the whole lenght of glass. Let it dry on air without washing.
- It is important to form thin layer of dye with adequately diluted number of cells.
- Observe at magnification 1000x with immersion oil.

Evaluation: Colourless cells are visible on dark background in preparation (fig. 46). Compare the shape and size of cells of various species of one bacterial genus. Result is affected by the thickness of dye layer (strong layer can chap after drying) and by the concentration of dye.

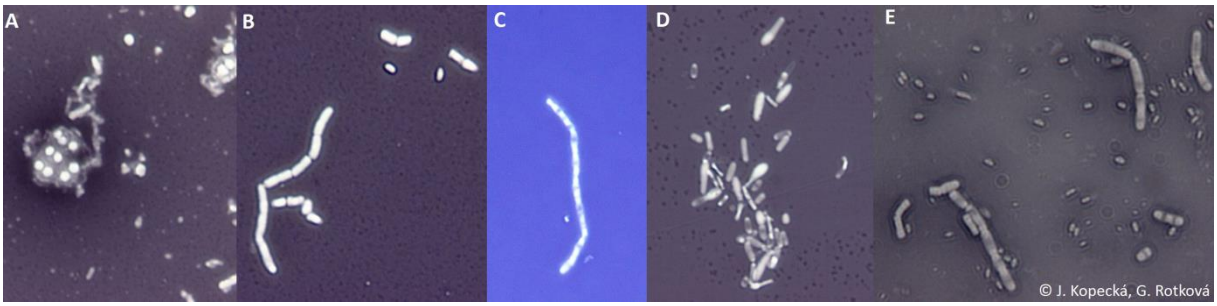


Fig. 46. Negative staining by nigrosin, *Azotobacter vinelandii* (A), *Bacillus megaterium* (B), *B. mycoides* (C), *B. sphaericus* (D), *B. thuringiensis* (E).

Negative staining by Kongo red

- Transfer cells aseptically into the small drop of Kongo red.
- Smear the drop gently by another microcopic glass (applied under angle of 45˚) over the whole lenght of glass. Let it dry on air without washing.
- Wash with 1 % of HCl, wash out the HCl immediately. Dry it by filter paper.
- Observe at magnification 1000x with immersion oil.

Negative staining of capsules (genus *Azotobacter*) by nigrosin

- Transfer the small amout of cells from mucilaginous colony in the drop of distilled water. Mix with drop of nigrosin and cover it by cover glass.
- Remove the rest of liquid by filter paper and observe at magnification 600x or 1000x.
- Grey cells are surrounded by white capsules, background is dark. Nigrosin stains only cells and background, not the capsules.

Negative staining of capsules (genus *Azotobacter*) by nigrosin, afterstaining of cells by methylene blue

- Mix the drop of nigrosin with drop of water. Transfer cells in suspensin, smear it over the glass and let it dry.
- Cover the smear by the methylene blue solution for 3 minutes, wash with water and dry by filter paper.
- Observe at magnification 1000x with immersion oil.

Evaluation: Blue cells are surrounded by light capsules, background is dark.

Negative staining of capsules (genus *Azotobacter*) by Kongo red, afterstaining of cells by methylene blue

- Transfer cells in drop of Kongo red, smear the suspension and let it dry.
- Cover the smear by HCl for several seconds. Pour off the acid, do not wash, dry by filter paper.
- Cover the smear by methylene blue for 3 minutes, pour off dye, wash with water and let dry on air.

- Observe at magnification 1000x with immersion oil.

Evaluation (fig. 47): Blue cells and cysts are surrounded by light capsules on dark background.

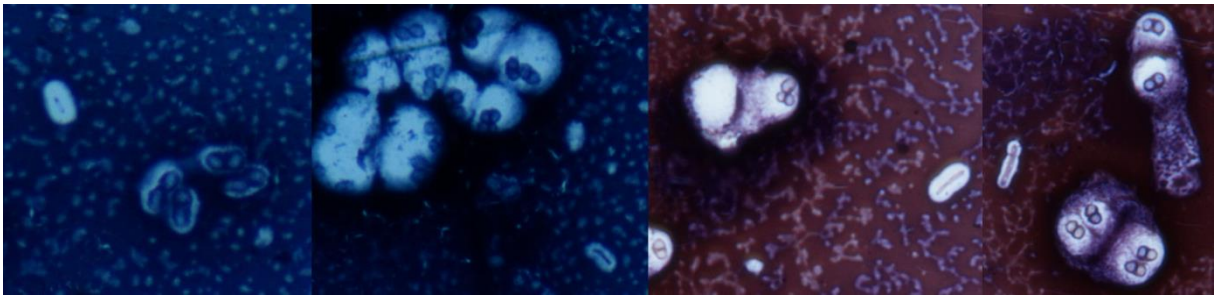


Fig. 47. Negative staining of genus *Azotobacter*.

Direct staining of capsules by hot carbolfuchsin

Evaporations of carbolfuchsin are toxic, this experiment is thus not performed in practice.

Native preparation for spores observing - genus *Bacillus* (fig. 48)

- Transfer cells in the drop of sterile distilled water, mix it and cover by cover glass (do not get the glass from upside, but at first with one edge, do not push).
- Observe the cells from liquid medium direct in medium without dilution in drop of water.
- Observe immediately by phase contrast (objective 60x or 100x), preparation is quick dryed out.

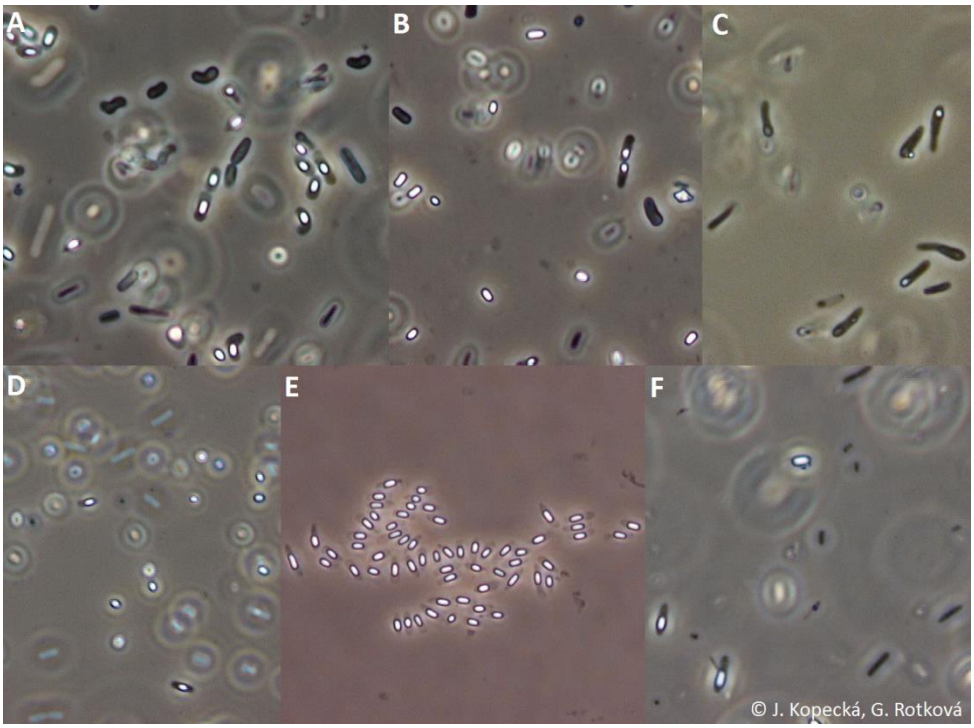


Fig. 48. The application of phase contrast in sporulating bacteria; *Bacillus cereus* (A), *B. megaterium* (B), *B. sphaericus* (C), *B. subtilis* (D), *B. thuringiensis* (E), *Paenibacillus polymyxa* (F).

Staining of spores by malachite green - Schaeffer-Fulton method

- Fix the dryied smear of cells on microcopic glaas by flame.
- Cover the smear by malachite green, warm 3 – 4times until evaporation for 5 minutes, supplement the dye continuously.
- Wash with water, cover it by contrast dye (Kongo red) for 1 – 3 minutes without warmingfor afterstaining.
- Wash with water, dry it, observe at magnification 1000x with immersion oil.

Evaluation: green spores inside red cells, the shape and location of spores can be evaluated inside cells and also in released spores (fig. 49).

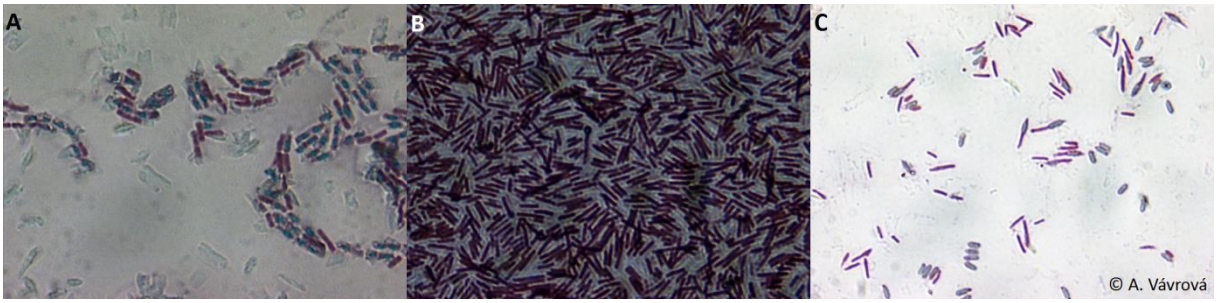


Fig. 49. Spores of *Bacillus cereus* (A), *B. sphaericus* (B) and *Paenibacillus alvei* (C) stained by Schaeffer-Fulton method.

Conclusion

Were the spores, capsula, cysts and cells good distinguishable and observable? Was the difference observed in size of capsules between wild type isolate (soil) and type strain (CCM)? Were the spores located at the same place in various cultures? Have the spores the same shape and size?

Additional information

Prescott L., M., Harley J. P., Klein D. A., Microbiology, WCB, Dubuque, 1996, ISBN 0-697-29390-4.

Songer J. G., Clostridia as agents of zoonotic disease. Vet. Microbiol., 2010, 140: 399-404.

The list of proteins comprising in proces of sporulation (http://www.uniprot.org/uniprot/?query=sporulation&sort=score, 8. 3. 2016).

Control questions

1. What part of the preparation do we stain by negative staining?
2. How we can highlight the capsule in preparation?
3. For what is important preparation stained according to Schaeffer-Fulton method?
4. What number of spores is present in bacterial cell, how we can them observe?
5. Where could be the spore located in the cell?
6. Could be the spores observed by phase contrast? If yes, how do they look like?
7. What is the germination?
8. Write some characteristics of spores (number, location, shape, structure).
9. Write at least 2 genera of sporulating bacteria.
10. What advantages gives negative stained preparation and native preparation for obseving of bacterial cell morphology?
11. What microscopically technique can we use for observing of spores?
12. Why is important shape and location of endospores in various species of genus *Bacillus*?
13. Why is staining of spores problematic?
14. Are the cells stained during the negative staining?

Basic microbiological analysis of water

Aim of practice

Determine total number of psychrophillic and mezophillic bacteria on universal medium and of indicator bacterial group on selective-diagnostic media from drinking water sample (water well, water) or surface water sample (pond, river, dam).

Introduction

Water is one of the natural environments of bacteria. Their amount and species diversity depend on carbon and nitrogen sources and oxygen presence. Number of microorganisms depends on environment characeristics (depth, presence of animals and plants, stream of water, pH, temperature, distance from waterside). Among **typical** water bacteria (**autochthonous** water bacteria**)** belong i.e. *Chromobacterium, Flavobacterium, Micrococcus, Sphaerotilus, Leptothrix, Spirillum*. If high amount of organic mattr is in water, anaerobic or optional anaerobic bacteria (i.e. *Clostridium*) could be presented in higher rate in water. Aerobic **soil bacteria**, i.e. *Bacillus, Micrococcus, Streptomyces* and coryneform bacteria *Corynebacterium, Brevibacterium* could be present in water due to the ablation of soil. Their occurence in water is limited by concentration of nutrients. Bacterial contaminants, **human and animal intestinal bacteria**, especially family *Enterobacteriaceae*, some streptococci (i.e. *Enterococcus faecalis*) and some species of genus *Clostridium* could be present in water for short time. Even pathogenic bacteria (i.e. *Salmonella* typhi, *Shigella dysenteriae*) could be isolated from water in certain conditions, water is not suitable place for them, pathogenic bacteria survive only limited.

Blue-green algae (*Cyanobacteria*) have gramnegative type of cel wall, occur commonly in water, soil, deserts and in polar regions. They photosynthetize, cells contain chlorophyll in thylakoids a fycobilizomes (other photosynthetizing bacteria contain pseudochlorophyll). They differ by presence of external coat composed from polysaccharides or polypeptides (glykokalyx), gas vacuoles and by presence of enzyme RUBISCO in carboxyzomes from other prokaryotes. Diferenced cells: heterocysts (N_2 fixation), akinetes (static stadia), baeocytes (reproduction). Phosphorus, higher temperature, certain pH and amount of nutrient are necessary for massive development of cyanobacteria in water. They could produce cyanotoxins and cause s.c. water bloom. Some strains: *Spirulina, Nostoc, Anabaena, Chroococcus, Cyanobacterium, Microcystis*.

Specific environment is sea and ocean for microorganisms. Microorganisms are part of plankton, participate on nutrient cycling, have to be tolerant to salt, tempperature and pressure, i.e. *Shewanella, Vibrio, Marinobacter, Colwellia, Thiomargarita namibiensis*. Extremophilic microorganisms (temperature, pressure) occur in deep-sea trenches.

Microbiological water analysis is a part of complex evaluation of water quality, its performance is regulated by state norm. All microorganisms including pathogenic microbes can not be determinated by water analysis because of material and time demands. **Assessment of s.c. indicator groups of bacteria** is used for evalution of harmful microorganisms from hygienic point of view that have the same ecological character and could be determinated quick and simple. Assessment of indicator groups (coliform bacteria, fecal coliform bacteria, enterococci, mesophilic bacteria, psychrophilic bacteria, colourless flagellates) show which microbial groups water contains. Drinking water can not contain any coliform bacteria, fecal coliform bacteria and enterococci. Coliform, fermenting intestinal bacteria are evidence of fecal contamination of water and could be indicator for pathogens presence. Indicator value of enterococci and coliform bacteria is not definite, is highly discussed. They are not yet satisfactory replaced as fundamental hygienic organisms.

Endo agar is selective-diagnostic medium with basic fuchsine and lactose that eliminates growth of grampositive bacteria. Utilisation of lactose is typical for fermenting coliform bacteria. Indicator is Schiff reagent that indicates acetaldehydes. Most of gramnegative aerobic, optional anaerobic bacteria grows on Endo agar. Colonies of lactose-positive bacteria growing in darkred colonies, *Escherichia coli* mostly with metallic gold lustre are counted at evaluation. Lactose-negative species grow in pink or colourless colonies, are not included to evaluation. Cultivation is performed at 37 °C.

Slanetz-Bartley agar is used for enterococci determination. Medium contains triphenyltetrazolium chloride that is reduced to red formazan (dark red colonies) by enterococci. Sodium azide serves as selective agent to inhibit growth of gramnegative bacteria. Cultivation at 44 °C is recommended because of growth of streptococci at 37 °C on medium. Another confirmatory test is utilisation of antibiotics to differentiate other streptococci. Red colonies or colonies with red middle and pink margin are counted at evaluation; white, colourless colonies or small red colonie (less than 2 mm) are not counted.

Agar mFC contains lactose. Selective agent for grampositive organisms are bile salts. Medium contains aniline blue that stains colonies in blue. Light pink and grey colonies are not counted at evaluation. Cultivation is performed at 44 °C.

TYEA agar contains tryptone, yeast extract and agar. It is universa medium (nonselective) that serve to assess total number of microorganisms (general contamination of water). All types of colonies are counted at evaluation. Cultivation is performed at 22 °C for psychrophilic and at 37 °C for mesophilic microorganisms.

Psychrophilic microorganisms have with growth optimum at 20 °C, indicate the presence of organic compounds that could be quickly utilised by bacteria at low temperatures. Their assessment is performed in drinking water, surface water in the case of processing to drinking water. Only their very high amount means the presence of many organic compounds in water. Psychrophilic bacteria are natural autochthonic in water.

Mesophilic bacteria have growth optimum at 37 °C, indicate contamination by warm-blooded animal and humans including possible pathogens. Only limited number is allowed in water.

Coliform bacteria are some species of gramnegative bacteria (family *Enterobacteriaceae*) that utilise lactose during 24-48 hours during cultivation on selective-diagnostic media. Typical representative is species *Escherichia coli* living mostly in digestive tract of warm-blooded animal including human. It is indicator of fecal contamination, grows up at 42-44 °C. Except for *E. coli*, species of genera *Enterobacter, Citrobacter* and *Klebsiella* belong to coliform bacteria. These bacteria could be present also in another environments beside digestive tract. Many nutrients media are used for coliform bacteria cultivation, mostly with content of lactose. Presence of coliform bacteria in water is evidence of fecal conatmination and thus possible contamination of intestinal pathogenic bacteria (*Salmonella, Shigella*). Basic water analysis have to be extended with pathogens analysis in the case of suspicion.

Assessment of **thermotolerant fecal coliform bacteria** using s.c. temperature test confirms **fresh fecal contamination** and differentiate odler contamination that is already part of heterotrophiccommunity. Test is performed at 44 °C on mFC medium.

Enterococci (i.e. *E. faecalis, E. faecium, E. gallinarium, E. avium*) are group of optional anaerobic grampositive streptococci that occurs in human and animal digestive tractu but alsoin environment. Enterococci grow in presence of sodium chloride (6,5 %), at basic pH 9,6 and at 10 - 45 °C compared to others streptococci. They show relatively higher thermoresistance nd resistance to another physical and chemical effects. They are considered as signifficant indicator of **fresh fecal contamination**, especially in drinking water adjusted by disinfection.

Rules for sampling before cultivation – microbiological analysis

Vessels have to be from colourless, transparent material (glass, polyethylene, polypropylene). Vessels have to sterilized in autoclave at 121 °C for at least 15 minutes or by dry sterilization at 160 - 170 °C for at least 1 hour or have to be declared as sterile by manufacturer. Aseptic principles of work have to be maintained by sampling. Samples have to be protected from light during transport and maintained in box or fridge at 4 °C before processing. Time between sampling and procesing have to be short, ideally in 24 hours (storege of sample in dark at 4 °C ± 3 °C).

Drinking water

Problems of drinking water deals public notic Vyhláška 252/2004 Sb. Evaluation of drinking water quality is performed by two types of analysis. Only some chemical and microbiological parameters are assessed by short analysis (well of private persons). Analysis is need to be extended about geological parameters in locations where uranium, arsenic, antimony is present. Well owners shloud control periodically quality of drinking water, analyses are performed by hygienic statiions or acredited firms. Short analysis (circa 1500 - 3000 CZK) contains assessment of microbes, colour, turbidity, chemical consumption of oxygen and very limited amount of chemical compounds (mostly compound containing nitrogen: nitrates, nitrites, ammonia. Complex water analysis (accoridng to public notice Vyhláška 252/2004 Sb.) is required by hygienic stations on public water-main operators, places of public catering, hotels with its own source of drinking water. Periodicity of analyses is regulated by relevant hygienic station. All data from measurement of water-mains, pools, public swimming pool have to be sended in drinking water database s.c. PiVo of Ministry of health of Czech Republic from year 2004.

Surface water

Observing and evaluation of water quality in watercourses and water tank and heads is managed by individual basins – Basin of Labe, Vltava, Odra, Ohře and Morava. Another measurements perform Czech Hydrometeorogical Institute, TGM Research Water Institute and State Ameliorative Management. Quality of water was improved by sewage disposal plants, limitation of fertilizer usage in agriculture and limitation of industrial productions. Microbial contamination from communal sources is still problematic. Microbiological parameters of waste water analyses are not required by valid public notice (Nařízení vlády 61/2003).

Instruments and microorganisms

TYEA (tryptone-yeast extract-agar)

Slanetz-Bartley (SB) agar, Endo agar, mFC agar

Physiological solution, pipettes, sticks, tubes

Sample of water (drinking/surface water)

Methods

Drinking water – we do not expect the contamination, **nondiluted sample and dilution 10^{-1}**.

Surface water – we expect the contamination, **dilution 10^{-1} and 10^{-2}**.

Always mention the source of water in protocol!

Every dilution innoculate on at least 2 dishes for calculation of arithmetic average (fig. 50).

Procedure in practice is based on norm ČSN 830521; today is valid another procedure according to Vyhláška č. 252/2004 Sb. (modification of media used to grow of target bacterial species; modificated media for another indicator bacterial groups after filtration of water).

General contamination

- Dilute the sample (nondiluted sample and 10^{-1} for drinking water; dilution 10^{-1} and 10^{-2} for surface water) according to type of sample (0,5 ml of sample in 4,5 ml of physiological solution).
- Mix the sample properly and pipette 1 ml of each dilution in sterile Petri dishes.
- Pour the samples with circa 15 ml of temperated TYEA medium.
- Cultivation is performed at 22 (psychrophiles) and at 37 °C (mesophiles).
- The total number of 8 dishes is needed for one sample (2 dilution, each on 2 dishes, temperatures of cultivations are 22 and 37 °C).

Assessment of indicators by selective-diagnostic madia

- Dilute the sample (nondiluted sample and 10^{-1} for drinking water; dilution 10^{-1} and 10^{-2} for surface water) according to type of sample (0,5 ml of sample in 4,5 ml of physiological solution).
- Mix the sample properly, pipette 0,1 ml of sample on agars (SB, ENDO and mFC agar) and smear it by the stick.

- Cultivation is performed at 37 °C (ENDO agar) and at 44 °C (SB and mFC agar).

- It is possible to concentrate the sample by filtration before the cultivation – assessment of enterococci from drinking water (SB agar).

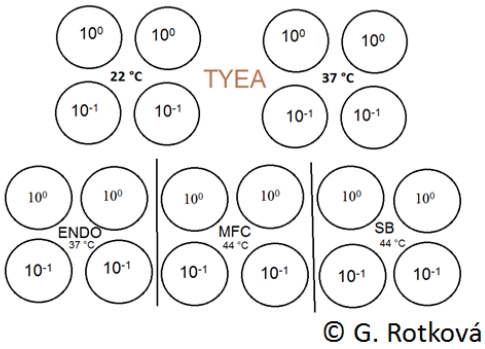


Fig. 50. Number of dishes for 1 sample by water analysis (dilution are mentioned for drinking water).

Conclusion

All types of growing colonies are counted on **TYEA agar** (fig. 51).

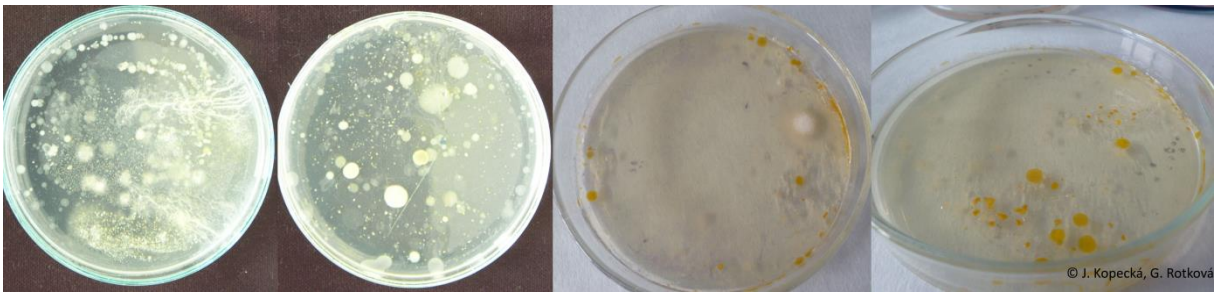


Fig. 51. Growth of bacteria on TYEA agar.

Colonies with metallic lustre of *E. coli*, dark-red colonies of other coliform bacteria are counted on **Endo agar** (fig. 52). Pink or transparent colonies are lactose negative, they are not counted.

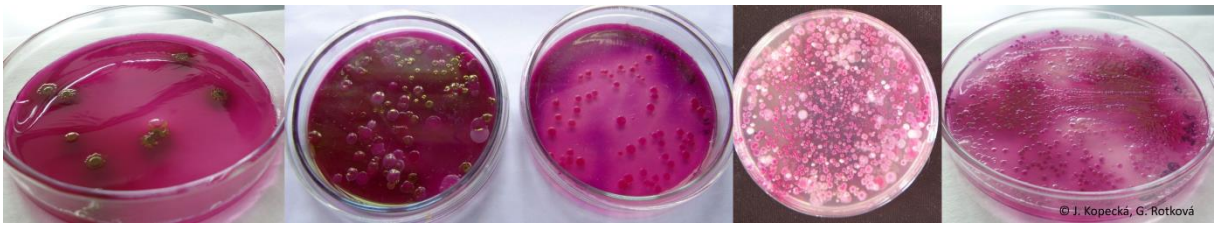


Fig. 52. Bacterial growth on Endo agar.

Dark-red colonies or colonies with red center and pink margin are counted on **SB agar** (fig. 53)

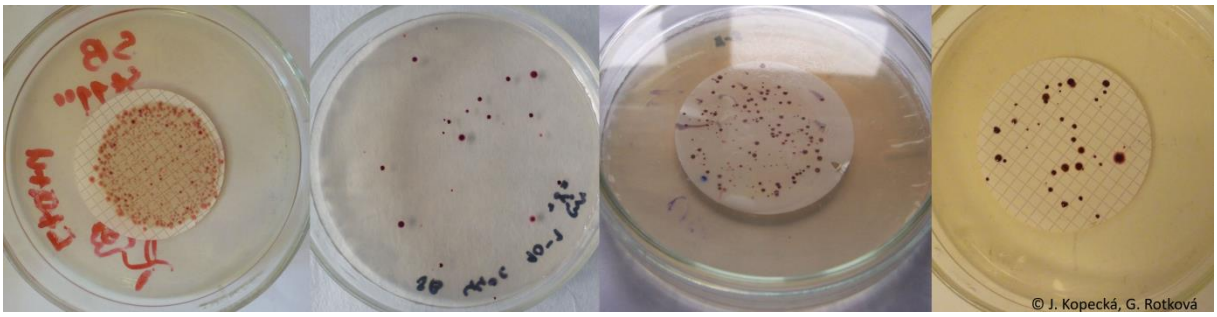


Fig. 53. Bacterial growth on SB agar.

Violet or dark-blue lactose-positive colonies are counted on **mFC agar** (fig. 54), light pink and grey colonies are not counted.

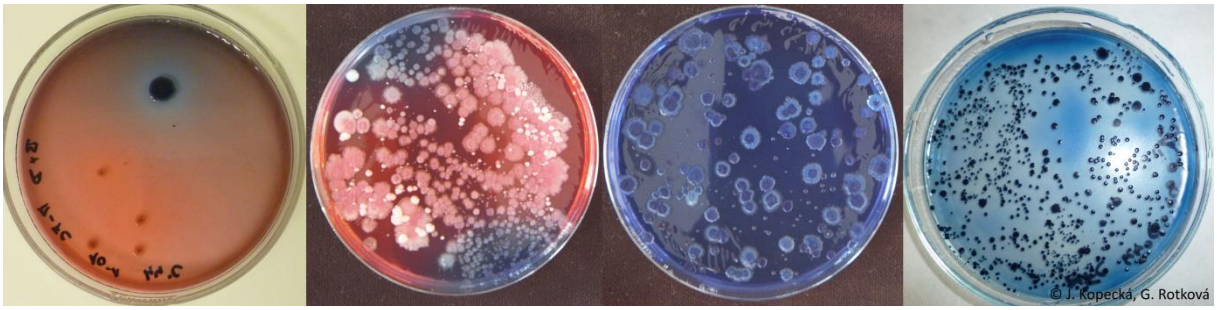
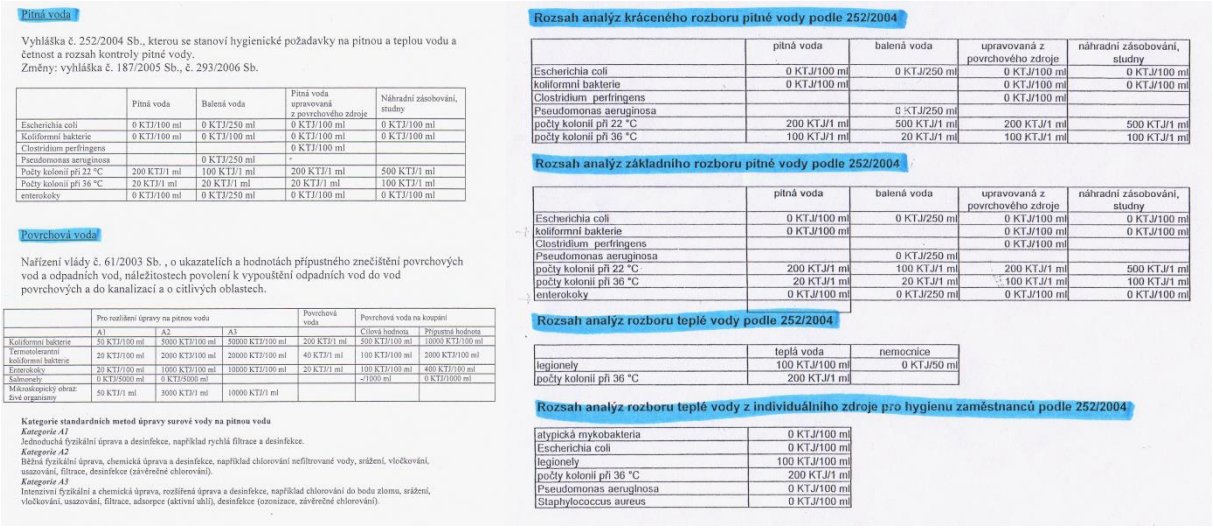


Fig. 54. Bacterial growth on mFC agar.

For results evaluation is necessary to recount the result in units **CFU/ml** of original sample. The appropriate dilutions (circa 20 - 200 colonies on dish) are selected for evaluation and the number of colonies is counted. From number of colonies, the average is calculate and multiplicate by value of dilution (positive value). When we innoculate only 0,1 ml of sample on the dish (selective media), we have to multiply the result 10 times (to get CFU/ml). Compare the result with tables for water analysis according to public notice - Vyhláška č. 252/2004 Sb. (fig. 55).



Pitná voda

Vyhláška č. 252/2004 Sb., kterou se stanoví hygienické požadavky na pitnou a teplou vodu a četnost a rozsah kontroly pitné vody.
Změny: vyhláška č. 187/2005 Sb., č. 293/2006 Sb.

	Pitná voda	Balená voda	Pitná voda upravovaná z povrchového zdroje	Náhradní zásobování, studny
Escherichia coli	0 KTJ/100 ml	0 KTJ/250 ml	0 KTJ/100 ml	0 KTJ/100 ml
Koliformní bakterie	0 KTJ/100 ml	0 KTJ/100 ml	0 KTJ/100 ml	0 KTJ/100 ml
Clostridium perfringens			0 KTJ/100 ml	
Pseudomonas aeruginosa		0 KTJ/250 ml		
Počty kolonií při 22 °C	200 KTJ/1 ml	100 KTJ/1 ml	200 KTJ/1 ml	500 KTJ/1 ml
Počty kolonií při 36 °C	20 KTJ/1 ml	20 KTJ/1 ml	20 KTJ/1 ml	100 KTJ/1 ml
enterokoky	0 KTJ/100 ml	0 KTJ/250 ml	0 KTJ/100 ml	0 KTJ/100 ml

Povrchová voda

Nařízení vlády č. 61/2003 Sb. , o ukazatelích a hodnotách přípustného znečištění povrchových vod a odpadních vod, náležitostech povolení k vypouštění odpadních vod do vod povrchových a do kanalizací a o citlivých oblastech.

	Pro rozlišení úpravy na pitnou vodu			Povrchová voda	Povrchová voda na koupání	
	A1	A2	A3		Cílová hodnota	Přípustná hodnota
Koliformní bakterie	50 KTJ/100 ml	5000 KTJ/100 ml	50000 KTJ/100 ml	200 KTJ/1 ml	500 KTJ/100 ml	10000 KTJ/100 ml
Termotolerantní koliformní bakterie	20 KTJ/100 ml	2000 KTJ/100 ml	20000 KTJ/100 ml	40 KTJ/1 ml	100 KTJ/100 ml	2000 KTJ/100 ml
Enterokoky	20 KTJ/100 ml	1000 KTJ/100 ml	10000 KTJ/100 ml	20 KTJ/1 ml	100 KTJ/100 ml	400 KTJ/100 ml
Salmonely	0 KTJ/5000 ml	0 KTJ/5000 ml			-/1000 ml	0 KTJ/1000 ml
Mikroskopický obraz: živé organismy	50 KTJ/1 ml	3000 KTJ/1 ml	10000 KTJ/1 ml			

Kategorie standardních metod úpravy surové vody na pitnou vodu
Kategorie A1
Jednoduchá fyzikální úprava a desinfekce, například rychlá filtrace a desinfekce.
Kategorie A2
Běžná fyzikální úprava, chemická úprava a desinfekce, například chlorování nefiltrované vody, srážení, vločkování, usazování, filtrace, desinfekce (závěrečné chlorování).
Kategorie A3
Intenzivní fyzikální a chemická úprava, rozšířená úprava a desinfekce, například chlorování do bodu zlomu, srážení, vločkování, usazování, filtrace, adsorpce (aktivní uhlí), desinfekce (ozonizace, závěrečné chlorování).

Rozsah analýz kráceného rozboru pitné vody podle 252/2004

	pitná voda	balená voda	upravovaná z povrchového zdroje	náhradní zásobování, studny
Escherichia coli	0 KTJ/100 ml	0 KTJ/250 ml	0 KTJ/100 ml	0 KTJ/100 ml
koliformní bakterie	0 KTJ/100 ml		0 KTJ/100 ml	0 KTJ/100 ml
Clostridium perfringens			0 KTJ/100 ml	
Pseudomonas aeruginosa		0 KTJ/250 ml		
počty kolonií při 22 °C	200 KTJ/1 ml	500 KTJ/1 ml	200 KTJ/1 ml	500 KTJ/1 ml
počty kolonií při 36 °C	100 KTJ/1 ml	20 KTJ/1 ml	100 KTJ/1 ml	100 KTJ/1 ml

Rozsah analýz základního rozboru pitné vody podle 252/2004

	pitná voda	balená voda	upravovaná z povrchového zdroje	náhradní zásobování, studny
Escherichia coli	0 KTJ/100 ml	0 KTJ/250 ml	0 KTJ/100 ml	0 KTJ/100 ml
koliformní bakterie	0 KTJ/100 ml		0 KTJ/100 ml	0 KTJ/100 ml
Clostridium perfringens			0 KTJ/100 ml	
Pseudomonas aeruginosa		0 KTJ/250 ml		
počty kolonií při 22 °C	200 KTJ/1 ml	100 KTJ/1 ml	200 KTJ/1 ml	500 KTJ/1 ml
počty kolonií při 36 °C	20 KTJ/1 ml	20 KTJ/1 ml	100 KTJ/1 ml	100 KTJ/1 ml
enterokoky	0 KTJ/100 ml	0 KTJ/250 ml	0 KTJ/100 ml	0 KTJ/100 ml

Rozsah analýz rozboru teplé vody podle 252/2004

	teplá voda	nemocnice
legionely	100 KTJ/100 ml	0 KTJ/50 ml
počty kolonií při 36 °C	200 KTJ/1 ml	

Rozsah analýz rozboru teplé vody z individuálního zdroje pro hygienu zaměstnanců podle 252/2004

atypická mykobakteria	0 KTJ/100 ml
Escherichia coli	0 KTJ/100 ml
legionely	100 KTJ/100 ml
počty kolonií při 36 °C	200 KTJ/1 ml
Pseudomonas aeruginosa	0 KTJ/100 ml
Staphylococcus aureus	0 KTJ/100 ml

Fig. 55. Selection of requirements on microbiological and biological analysis of drinking and surface water in the Czech Republic (Vyhláška č. 252/2004 Sb.).

Additional information

Baudišová D., Správnost a přesnost výsledků při mikrobiologických analýzách vody, VÚV, 1998 (http://www.mzp.cz/ris/ais-ris-info-copy.nsf/4d735ff9c7e64b58c12569e7001a2d9c/3e5d5bba 30e14c0a8025680e003396c6?OpenDocument, 13. 3. 2016).

Centrum pro cyanobakterie a jejich toxiny (www.sinice.cz, 13. 3. 2016).

Fykologická laboratoř Přírodovědecké fakulty Jihočeské univerzity (www.sinicearasy.cz, 13. 3. 2016).

Kvalita pitné vody (http://sweb.cz/Hlavaty.Vaclav/kvalita.htm, 13. 3. 2016).

Vyhláška č. 252/2004 Sb. (http://sweb.cz/Hlavaty.Vaclav/vyhl.htm, 13. 3. 2016).

Control questions

1. What could be the inaccuracies during the microbiological water analysis?
2. What is the difference of drinking and surface basic microbiological water analysis?
3. What is the procedure to estimate the total number of psychrophillic and mesophillic bacteria in the sample of drinking and surface water?
4. What selective-diagnostic media we can use during the basic microbiological water analysis? Do we count all colonies on them?
5. What is the bacterial indicator group for basic microbiological water analysis?
6. Utilisation of what sugar is monitored on Endo agar?
7. Do all bacterial colonies count on the selective-diagnostic media?
8. Write at least 4 species of water bacteria.

Identification of bacteria, biochemical tests and standardised identification systems

Aim of practice

Identification of unknown sample by the phenotype characteristics. Choice of suitable strain for identification system ENTEROtest 16.

Introduction

The basic prerequisite of right identification is manipulation with pure culture and its basic cultivation for sample propagation. Cultivation on diagnostic media helps to determinate approximate taxonomic group in some samples.

Identification of unknown bacterial samples is grounded on evaluation of **morphology** (macroscopic and microscopic characteristics, character of growth and colonies, identification staining – Gram, acid-fast, spores and capsules formation), **physiology** (relationship to oxygen, growth temperature, resistance and tolerance to NaCl, bile acids, antibiotics, type of metabolism) and **biochemical characteristics** (source of carbon, nitrates reduction, indol formation). If these tests do not lead to succesfull identification, additional tests could be used, i.e. serotypisation, phagotypisation, immunochemical reaction, chemical analysis of cell wall, toxins production, specific antigens, aglutination or genotypisation - PCR, sequencing of 16S rRNA, ribotypisatione, hybridisation.

Tests are divided in several groups: slide (KOH test, katalase test), tube (OF test, TSI test), paper (ONPG test, oxidase test), microtesta (ENTEROtest). Plate microtests save material, energy, time, space, are simple to use compared to the tube tests (one test = one tube). Microtest contains the representative set of 10 - 20 biochemical tests. Microtests help to eliminate inconsistency in individual differences in media preparation, procedure and evaluation. Diagnostic tables and computer programmes are used to evaluation. Tests have long-term storage capacity. Mikro-LA-Tests (developed and produced by concern Lachema in cooperation with Czech Collection of Microorganisms) are mostly used for bacterial identification: for gramnegative fermenting rods (family *Enterobacteriaceae*) is used ENTEROtest and ENTERO Rapid, for anaerobes ANAEROtest, for staphylococci and micrococci STAPHYtest, for streptococci and enterococci STREPTOtest, for gramnegative nonfermenting rods NEFERMtest.

Example of unknown sample identification

1. KOH test – differentiation between grampositive and gramnegative cells
2. Cultivation on MPA and blood agar, evaluation of colony appearance, temperature of cultivation
3. Gram staining, microscopy
4. Grampositive cells: shape of cells and another characteristics
    i. rods – spores staining – sporulating (shape and location differ in genus *Bacillus*), nonsporulating (*Lactobacillus*)
    ii. cocci – morphology of clumps, grapes (*Staphylococcus*), pairs, tetrads (*Micrococcus*), packets, sarcins (*Sporosarcina*), chains (streptokoky).
    iii. katalase test – positive i.e. in genus *Staphylococcus*
5. Gramnegative cells: shape of cells (gramnegative cocci are less often, mostly pathogens) and another characteristics
    i. OF test - fermenting bacteria (optional anaerobic metabolism, i.e. enterobacteria); bacteria with respiratory metabolism (aerobic vibria, aeromonades).
    ii. Oxidase test for elimination vibria and aeromonades

Family ***Enterobacteriaceae*** is the most clinical important family (circa 65 species) of gramnegative rods (1-6 μm * 0,3-1 μm) that is very important also for nonclinical parts of microbiology. Enterobacteria do not form spores or cysts; immobile or peritrichous, chemoautothrophic bacteria. Growth at 18 – 40 °C with optimum at 37 °C. Family contains optional anaerobic species, most of them lives in digestive tract of vertebrates as part of normal microflora. Most strains are nonpathogenic, some species are pathogenic agents, *Yersinia pestis*, s.c. antropopathogenic serovars of *Salmonella* (serovars Typhi, Paratyphi A, Paratyphi B, Paratyphi C) and some strains of *Escherichia coli*. Intestinal enterobacteria are spreaded together with feces, their presence (i.e. in water) indicate fecal contamination. Genera are distinguised according to fermentation tests, reduction of nitrates, sulphan formation, urea hydrolysis, KCN-test, glycerol test, indol formation. Diagnostical media for enterobacteria are i.e. Endo and MacKonkey agar, desoxycholate-citrate agar, Salmonella-Shigella agar, agar with brilliant green, Simmons-citrate agar. They are oxidase negative (except for genus *Plesiomonas*), form katalase, utilise always glucose and form gas, reduce nitrates.

Tests used in practice

KOH test serves for quick differentiation of grampositive and gramnegative bacteria. Potassium hydroxide disrupts thin peptidoglycan layer of cell wall in gramnegative bacteria. Cell lysate forms viscous suspension that is spreaded after loop. It can not be used in bacteria producing mucus.

Katalase is enzyme decompoding hydrogen peroxide to free oxygen and water. Some bacteria are capable to reduce oxygen to hydrogen peroxide that is toxic for cells. Defensive mechanism decreasing this damage is based on decomposition of hydrogen peroxide to water and oxygen.

Medium with glucose and acid-base indicator bromthymol blue is used for **OF test, oxidation/fermentation test** (aerobic/anaerobic metabolism). Production of acids is part of glucose utilisation, the yellow colour (positive reaction) is formed in medium, indicator becomes blue by alkalisation in basic environment, green colour of medium is by neutral reaction. Medium in 2 tubes (without paraffin for aerobic conditions,with paraffin for anaerobic conditions) is innoculated by stab, bacterial motion could be readed in columnn also.

Positive oxidation and fermentation of glucose (both tubes are yellow) occurs in optional anaerobic microorganisms. Positive oxidation and negative fermentation of glucose is typical for aerobic nonfermenting species, i.e. *Aeromonas, Vibrio, Pseudomonas.* Negative oxidation and fermentation of glucose is typical for species that do not utilise glucose at all. Glucose enters in cell, is catabolised. Some microorganisms catabolise glucose oxidatively and form CO_2 and water. Most of them utilise glucose by fermentation. Microorganisms are capable ferment except for glucose ferment another monosaccharides, disaccharides or polysaccharides. Final products of fermentation are small organic molecules, commonly organic acids (i.e. lactic acid). Some microorganisms form gas during fermentation (hydrogen, CO_2). Formation of acids and gas is tested by fermentation tube.

TSI test (tripple-sugar iron) is used to determinate of lactose, glucose, sucrose utilisation, H_2S and gas production due to iron indicator according to Hajna. Acid-base bromocresol red serves as indicator, indicator for production of H_2S is iron. Some bacteria release hydrogen sulphide from aminoacids containing sulphur. Some enterobacteria form hydrogen sulphide by reduction of oxygen sulphur compounds. Production of hydrogen sulphide is detected by ferrous sulphate (black colour). Bubbles are created by gas production during fermentatin in tube. Medium is innoculated by stab and over the slant part of agar in upper side. Utilisation of glucose is evaluated in column, utilisation of lactose and sucrose in slant part of agar.

Oxidase test identificate organisms that create cytochrom c oxidase (last enzyme of respiratory chain) participating in electrons transport in electron transport chain of aerobic bacteria to oxygen. Oxidase agent contains chromogenic oxidation-reduction agent that changes colour by oxidation in blue or dark violet. Test is used for differentiation of species genus *Pseudomonas, Alcaligenes, Flavobacterium*. Oxidase positive are i.e. *Pseudomonas aeruginosa, P. putida, P. fluorescens, Plesiomonas*.

ONPG test proves production of ß-galactosidase that cleaves lactose. Colourless o-nitrophenyl-ß-D-galactopyranosid is hydrolysed to yellow ortho-nitrophenol in case of positive reaction.

ENTEROtest 16 is used to identify fermenting (positive fermentation test, negative oxidase test) strains of family *Enterobacteriaceae* isolated on i.e. Endo agar. It is commercial system of 16 well tests in 2 lines. Six strains could be identified on one plate. Test is supplemented by paper ONPG test (presence of β-galactosidase). Confirmation of family is performed by glucose fermentation test. Family *Vibrionaceae* is eliminated by oxidase test, vibria or aeromonades are oxidase positive.

The most frequently reasons for wrong identification are: contamination of sample, inappropriate density or suspension volume, pipitting of inoculum in another wells or reaction agent in another well, atypical strain or species that is not included in table.

Tests in wells: hydrogen sulphate production (H_2S), lysin decarboxylation (LYS), indol production (IND), ornithine decarboxylation (ORN), urea decomposition (URE), phenylalanine deamination (PHE), esculin hydrolysis (ESC), citrate utilisation (SCI), malonate utilisation (MAL), acid production from inositol (INO), adonit (ADO), cellobiose (CEL), sucrose (SUC), sorbitol (SOR), trehalose (TRE), mannitol (MAN).

Mutants are commonly present in environment, actualised tables containing percent of strains of target species that has positive reaction. Eventually, if result varies in cells of one strain. Results are written (+ or -) under corresponding reactions (fig. 56). Each reaction has mentioned the figure under its designation that erves for calculation of numaric code. If the reaction is positive, add the value of figure. If the reaction is negative, the value of figure is not subtracted. Numeric codes serve for more simply evaluation and better orientation in tables. Automatised reader could be used for results reading with subsequent evaluation due to corresponding software, eventually.

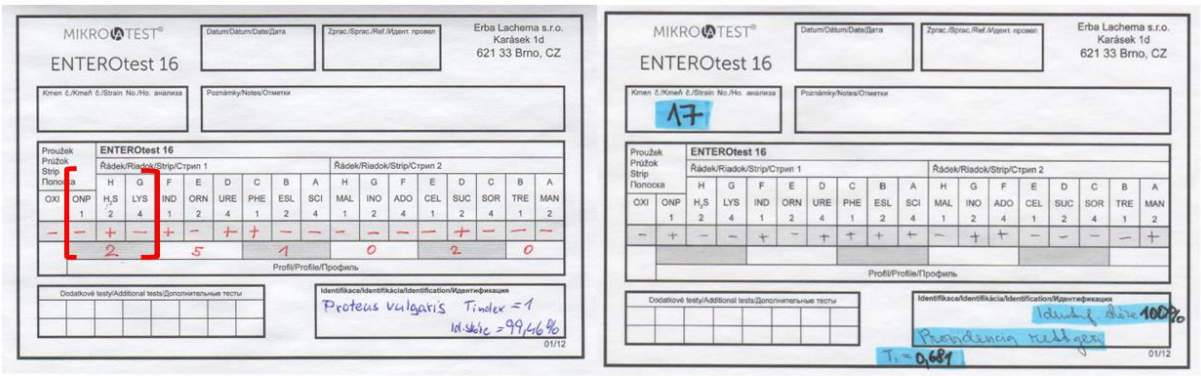


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Fig. 56. Evaluation of ENTEROtest 16.

Programme TNW is used for ENTEROtest evaluation. It could calculate identification score and T index in target result/strain. Identification score is degree of identification exclusivity, percent of probability that strain belongs to specific taxonu (species) and not to another: >99 % excellent differentiation; 96-99 % very good differentiation; 90-96 % strain is differentiated; < 90 % strain is not differentiated. T index determinates typicalness of identified strain to target taxon. The more higher T index, the more similar is strain to type strain of target taxon: >75 typical strain; 0,5-7,75 lower typical strain; 0,25-0,5 untypical strain; <0,25 completely untypical strain. Programme TNW suggests another differentiating tests for untypical strain or shows nonstandards results.

Control bacterial strains that have standard results are used for quality control of chemical used and for control of interpretation of colour reaction. Control strains are deponned by Czech Collection of Microorganisms in lyophilised culture or in gelatiniform discs.

It is necessary to assess unknown isolates complex – character of colony growth, optimum growth temperature, origin of sample, type of material, manipulation with sample.

Instruments and microorganisms

Sterile distilled water, physiological solution

Innoculation loops, microscopic slide, automatic pipettes

Microtest ENTEROtest 16

Oxidase and ONPG papers

3 % of hydrogen peroxide, 3% KOH

Unknown bacterial strain

Methods

The combination of tests is used in practice, some of the tests are only for demonstration (only results reading).

KOH test

- Transfer culture in drop of 3% KOH on microscopic slide.
- If cultura form viscous suspension after smearing by the loop and is drawing after the loop (damage of cell wall, pouring of cell content), the culture is gramnegative (fig. 57).

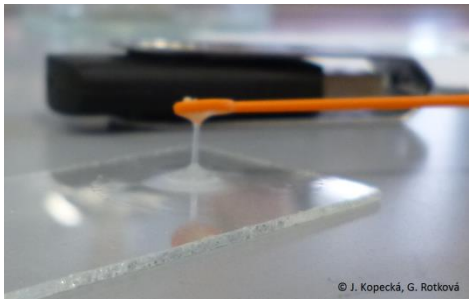

Fig. 57. Performance of KOH test in gramnegative bacteria (viscous suspenzion spreading after innoculation loop).

Catalase test

- Transfer culture in drop of 3% H_2O_2 on microscopic slide.
- Releasing of oxygen bubbles means positive reaction (fig. 58)

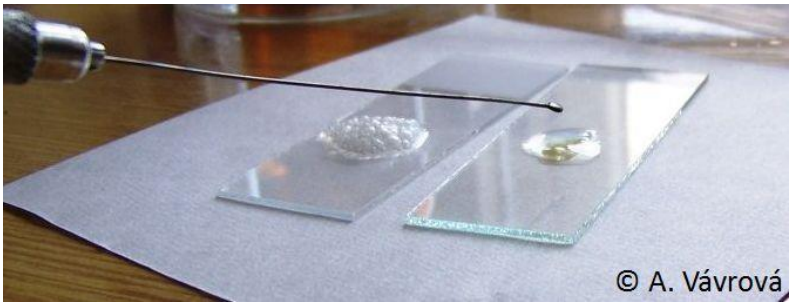

Fig. 58. Performance of catalase test.

OF test, oxidation/fermentation test (aerobic/anaerobic metabolism)

- Test is demonstration only for results reading. Two tubes are preparated for each strain, one with paraffin (anaerobic environment – fermentation of glucose), one without paraffin (aerobic environment – oxidation of glucose).
- Yellow colour means positive reaction; blue and green colours of medium mean negative reaction (fig. 59). Bacterial movement coould be read in the column also.

Fig. 59. Evaluation of OF test. Both reactions positive (yellow) – strain utilizes glucose by oxidation and fermentation; positive aerobic reaction, negative anaerobic reaction – strain utilizes glucose only by oxidation; both reaction negative (blue) – strain does not utilize glucose by oxidation and fermentation.

TSI test (tripple-sugar iron)

- Test is demonstration only for results reading.
- Medium is innoculated by the stab and on the surface (slant part of medium) in one tube.
- Changes of colours are observed (fig. 60). If culture ferments only glucose, only the column get yellow and the slant part remains red (i.e. *Salmonella*). If culture ferments glucose together with lactose or sucrose, column and the slant part is yellow. The black colour means production of H_2S. Gas formation is indicated by breaking of agar or its rising in tube.

Fig. 60. Evaluation of TSI test.

Oxidase test

- Transfer culture (24 hours old) on the OXI paper by the loop or put the OXI papaer direct on the culture on dish.
- Medium for bacterial growth dos not contain glucose or nitrates.
- If the reaction area gets blue in 30 seconds intensively, reaction is positive; I fit gets blue in 2 minutes, reaction is delayed positive. If the colour does not change ort he colour is changer after 2 minutes, reaction is negative (fig. 61).

Fig. 61. Oxidase test.

ENTEROtest 16

- I tis necessary to choose the appropriate strain for ENTEROtest, strain possible belonging to the family *Enterobacteriaceae* (gramnegative, catalase positive, fermentation and oxidation of glucose, oxidase negative).
- Prepare suspension of cells (from 24 hours old culture) in 3 ml of physiological solution with turbidity value 1 according to MacFarland scale.
- Plate for ENTEROtest is oriented according to manual, pull off the cover foil and sign the strip with number of strain.
- Pipette 100 μl of well mixed suspension in every well.
- Some tests are performed in anaerobic conditions, drop the corresponding wells with 2 drop of sterile paraffin oil: hydrogen sulphide test (H_2S), lysin (LYS), indol (IND), ornitin (ORN), urease (URE).
- Put the tests in bag before cultivation to aviod drying.
- Cultivation is performed at 37 °C for 18 - 24 hours together with ONPG test.

- Drop the well IND by reaction agent for indol and well PHE by reaction agent for phenylalanine aacording to manufacturer instruction before evaluation.
- Evaluate all reaction of ENTEROtest (fig. 62), phenylalanine in 3 minutes, because the positive reaction disappear after 3 minutes. Compare results with interpretation table (fig. 63) enclosed to the ENTEROtest (alternatively according to coloured comparing scale).

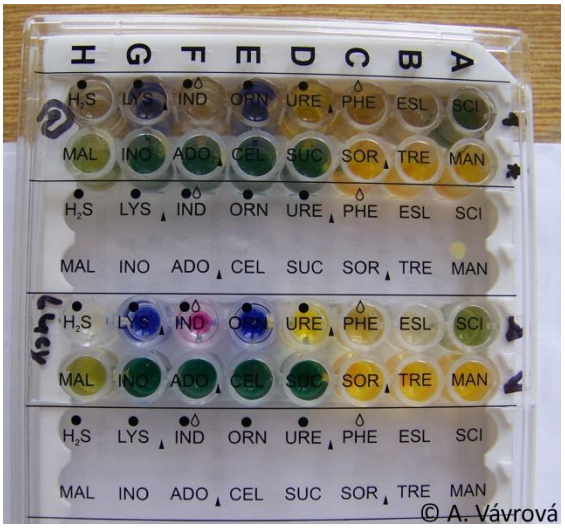

Fig. 62. ENTEROtest 16.

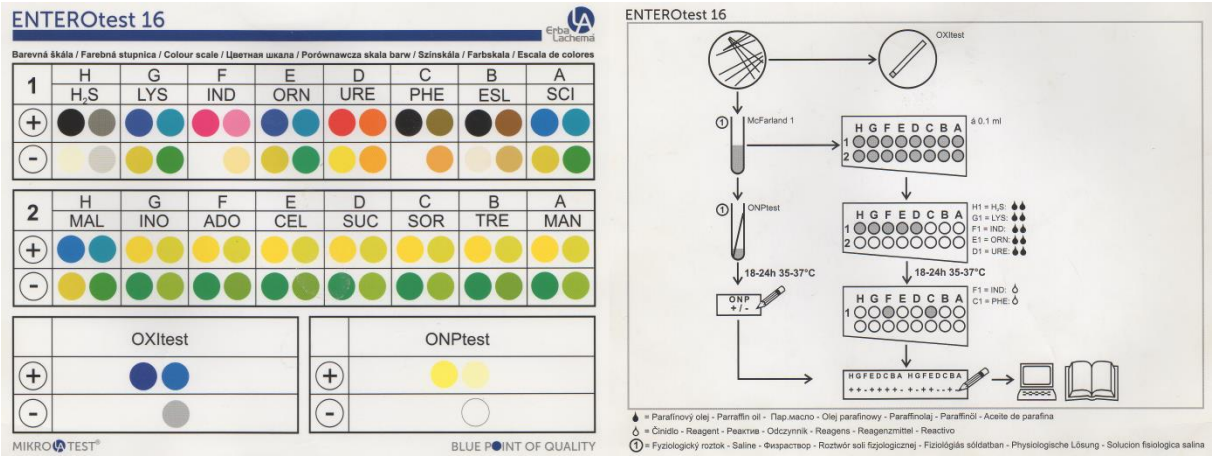

Fig. 63. Interpretation table for ENTEROtest 16 (Manual MikroLaTest, Erba Lachema).

ONPG test

- Put aseptically the ONPG test in the rest of suspension after performance of ENTEROtest.
- Cultivation is performed together with ENTEROtest for 24 hours at 37 °C.
- Positive reaction is clearly yellow colour (fig. 64).

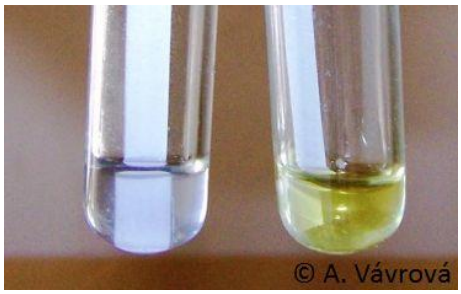

Fig. 64. Test for production of ß-galactosidase.

Conclusion

Did you succeed in identification of unknown sample?

Additional information

Systém MIKROLA (https://www.erbalachema.com/produkty-a-reseni/mikrobiologie/ system-mikrola/, 21. 3. 2016).

Czech Collection of microorganisms (http://www.sci.muni.cz/ccm/, 21. 3. 2016).

Control questions

1. For what serves KOH test?
2. Why is the KOH test used instead of Gram staining to differentiate grampositive and gramnegative cells?
3. What will happen, when we use bacterium from the unsuitable bacterial group (i.e. nonfermenting) for identification with ENTEROtest?
4. What is important fro the right identification of unknown sample?
5. What are the advantages of microtests compared to tube tests?
6. Utilisation of what sugar is monitored during the OF test?

PRACTICE FROM CYTOLOGY AND MORPHOLOGY OF BACTERIA

Gram staining, negative staining, native preparation

Aim of practice

Staining and observing of microscopical preparations.

Introduction

Native preparation

Cells are never fixed for observing by native preparation. Preparation is nonstained, serves for finding out the real shape and structure of cell that are not damaged by fixation and dying. It is used for observing of growth, reproduction and bacterial motion. It is important for study of cell structure and departments that are stained with difficulties, i.e. spores. Brief field, phase or Nomarski contrast are used to observe structures.

Fixation of preparation

The nature of fixation is precipitation of cell coloids, especially proteins. Cells adhere more easily to glass, are not washed away by application of dye or solvent and absorb dye better. Preparation is fixed when is completely dry to avoid cell boiling. Fixation is performed by pulling the glass through the flame, smear of cell sis located on the upper side of glass. If cells were cultivated in liquid sugar medium, it is necessary to separate cells from medium by centrifugation and wash them by water of buffer. Yeasts or micromycetes are bigger than bacterial cells, thermal fixation could change their real shape. Fixation is performed by chemikcals. Fixation and dying deform cells gentle, but their characteristic shape still remains. Nonfixed preparation negative stained (staining of backgrounf) is used for measurement of accurate size of cells.

Stained preparations

Stained preparations serve for estimation of cell wall type, shape of cells and their clusering, presence and location of spores, presence of capsules and internal structures (inclusions) and viability of cells. The simple staining of cell wall serves for evaluation of cell morphology and characteristic clustering of cells, i.e. staining by crystal violet without differentiation of grampositive and gramnegative type of cell wall. Vital staining differentiate dead cells because of dead cells absorb dye effectively and do not exclude it by efflux systems. Cell structures are differentiated by differentiation staining, internal and extrnal morphological

objects (spores, capsules, cell walls), chemical compounds (volutin, glycogen, starch). Diagnostic staining helps to identification of bacteria (i.e. Gram, acid-fast staining by carbolfuchsin, staining according to Giemsa). Cells are not fixed and stained by negative staining, only the background is stained. Negative staining is used for measurement of exact size of cells.

Preparation is always fixed before staining excepto for negative staining and vital test. Diluted water solution of organic dyes, usually salts, are used for staining. Basic dyes have coloured cationt, acidic dyes aniont. Basic dyes (i.e. crystal violet, methylene blue, safranin, basic fuchsin, malachite green) are mostly used. Staining could be highlighted by chemical treatment of cells (by phenol or tannin), chemicals serves as intermediate with higher affinity to cell and dye at the same time compared to affinity of cell to dye.

Gram staining

Gram staining is one of the most important diagnostic methods for bacteria identification. It differentiates groups of grampositive (stained bluu-violet) and gramnegative cells (stained red or pink) and specify some physiological and chemical characteristics of cell. The basic nature of Gram staining was not yet satisfactory explained. Differences in cell wall composition play the key role with high probability. Fixed preparation is stained by crystal violet aand Lugol solution (iodine in solution of KI). Complex of dye-iodine-cell wall is originated. Difference arises during the washing preparation by organic solvent (acetone or alcohol). Solvent solves the outer lipopolysaccharides layer, complex of crystal violet and iodine is washed away through thin peptidoglycan layer and cells are decolorised in the case of gramnegative cells. Grampositive cells maintain the dye. Cells are afterstained for highlighting the difference by another contrast dye (basic safranin, carbolfuchsine). Grampositive cells have binded crystal violet in cell wall that was not washed away by alcohol and stay blue-coloured. Faults during staining: too dense layer of cells; boiling cells during fixation; too long washing of cells by alcohol.

Gram staining is affected by physiological state of cells, age of culture and composition of medium. Cultures 24 hours old are the most suitable for staining. Cells can loose their grampositivity i.e. by mechanical damage, UV radiation, by acidic, basic or solvent treatment. Microorganisms that are stained partly as positive and partly as negative, are designated as gramlabile/gramvariable. Some of bacterial genera can not be stained by Gram staining – genera without cell wall (mycoplasma), spiral bacteria and strong acid-fast genera (mycobacteria); i.e. *Borrelia burgdorferi, B. recurrentis, Bartonella henselae, Chlamydia*

trachomatis, C. pneumoniae, Chlamydophila psittaci, Coxiella burnetii, Ehrlichia chaffeensis, Anaplasma phagocytophilum, Legionella sp., *Leptospira* sp., *Mycobacterium bovis, M. tuberculosis, M. avium, M. intracellulare, M. kansasii, M. leprae, M. marinum, Rickettsia rickettsii, Orientia tsutsugamushi, Treponema pallidum*.

Cell wall of bacteria

Cells of gramnegative type (obr. 65B) contain cell wall composed from outside lipopolysaccharide membrane and inside relatively thin peptidoglycan (approximately 5-10 % of cell wall) layer that contains muramic acid. Lipoproteins establish the binding between peptidoglycan and outside membrane. Lipopolysaccharides are composed from lipid A, core polysaccharid and O-antigen (O-chain). Outside membrane serves as protective barrier to environment, defends of permeability of compounds or reduces the speed of permeability (salts of bile acids, antibiotics, poisons).

Outside membrane lacks and peptidoglycan layer is relatively thick in grampositive type of cell wall (fig. 65A). Some bacteria can have teichoic acid, lipoteichoic acid or neutral polysaccharides in cell wall, or even mycolic acids.

Special group is bacteria without cell wall, s.c.. mycoplasma that are not able to synthetise the precursors of peptidoglycan. Cells are coated only by cytoplasmic membrane.

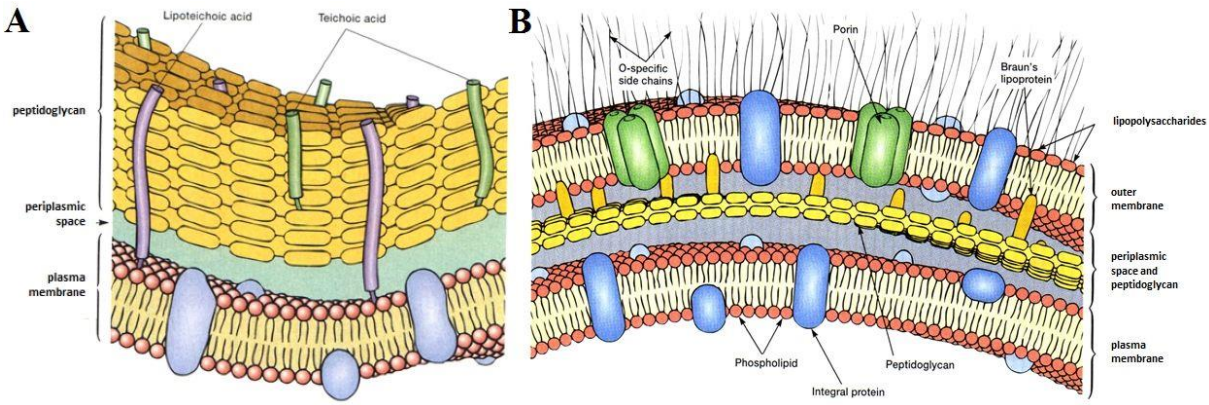

Fig. 65. Cell wall of grampositive (A) and gramnegative (B) type (Prescott et al., 1996, adjusted).

Negative staining

Only the background of cells is stained by negative staining, not cells. Size and real shape is not affected by fixation and dying. It is used for measurements of real size and shape of bacterial cell, capsules and mucus.

Characteristics of some microorganisms

Haloarcula – extreme pleomorphic rods, mostly flat, triangle- and square-shaped or irregular discs; gramnegative; extreme halophilous (2-5,2 M NaCl); bacterioruberins and yellow pigments

Haloferax – extreme pleomorphic, mostly shape of flat discs or cups; gramnegative; mucous colonies, extreme halophilous (1,5-4,5 M NaCl); bacterioruberina and yellow pigments

Sporosarcina – round or oval cells clustered as diplococci, tetrads or sarcina; grampositive; mobile by few flagella; spores formation; creamy or orange colonies

Leuconostoc – spherical or ovoid cells clustered in pairs or chains, sometimes short rods in long chains; grampositive

Azotobacter – big ovoid and pleomorphic cells, single, pairs, irregular clusters or in chains; cysts formation; gramnegative

Yarrowia lipolytica – yeasts, reproducing by vegetative buding; pseudohypha, hypha; stained as grampositive bacteria

Saccharomyces cerevisiae – yeasts, ellipsoidal, round cells; formation of branched pseudomycelium in some cases; mycelium do not form; reproducing by budding; haplo-diplobiotic life cycle; stained as grampositive bacteria

Instruments and microorganisms

Bacilli, G+ rods of various shapes (48 hours cultures with spores – phase contrast)

	Bacillus thuringiensis CCM 19
	Bacillus sphaericus CCM 1615
	Bacillus mycoides CCM 145
	Bacillus cereus CCM 2010
	Bacillus megaterium CCM 2007
	Bacillus subtilis CCM 2216
Archaea	*Haloarcula hispanica* CCM 3601^{T}
	Haloferax mediterranei CCM 3361^{T}
Eukaryota	*Saccharomyces cerevisiae*
	Yarrowia lipolytica
G- rods	*Serratia marcescens* CCM 303
	Escherichia coli CCM 3954
	Pseudomonas putida

G+ cocci, short rods	*Azotobacter vinelandii* CCM 289 – mucilaginous colonies, capsules
	Leuconostoc mesenteroides CCM 1803 – chains, capsule, mucus
	Sporosarcina ureae CCM 860
	Staphylococcus aureus
	Micrococcus luteus CCM 169

Crystal violet, Lugol solution, safranin

Nigrosin, Kongo red, methylene blue, HCl

Methods

Native preparation

- Drop sterile distilled water on microscopic slide.
- Transfer small amount of cells in drop of water by singed, cooled innoculation loop, mix it. Only small amount of culture is sufficient for avoiding dense preparation.
- Do not smear the drop. Cover the drop by cover glass without air bubbles formation (do not cover glass from upside, at first by one edge, do not push).
- Dry the remaining liquid by filter paper.
- Observe the cells from liquid medium direct in medium without dilution in drop of water.
- Choose the objective with phase contrast (Ph) for native preparation.
- Observe the preparation in 5 minutes for quick drying.

Simple staining

- Fix the dryed smear of cells by flame.
- Stain it in solution of crystal violet or safranin for 15-20 seconds.
- Wash it with water, dry it and observe at magnification 1000x.

Gram staining (fig. 66)

- Put out the cleaned microscopic slide from alcohol and pull it through the flame.
- Drop sterile distilled water in the middle of microscopic slide.
- Get a little amount of culture into the drop of water by singed, cooled loop and mix it.
- Smear the suspension onto the glass, let it dry and fix it by the flame (pull the glass through the flame for three times).

- Put the preparation in the solution of crystal violet (30 seconds), wash the dye by water.
- Put the preparation in the Lugol solution (30 seconds), wash the dye by water.
- Wash the preparation by ethanol (or acetone), max. for 15-20 seconds.
- Was the preparation by water.
- Put the preparation in the safranin for 1 minute (only gramnegative cells are stained by safranin, because crystal violet was decoloured; it is necessary to stain every preparation by safranin – we no not know how type of cells is in the preparation).
- Dry preparation by filter paper and observe at magnification 1000x (immersion objective) in brief field (BF objective).

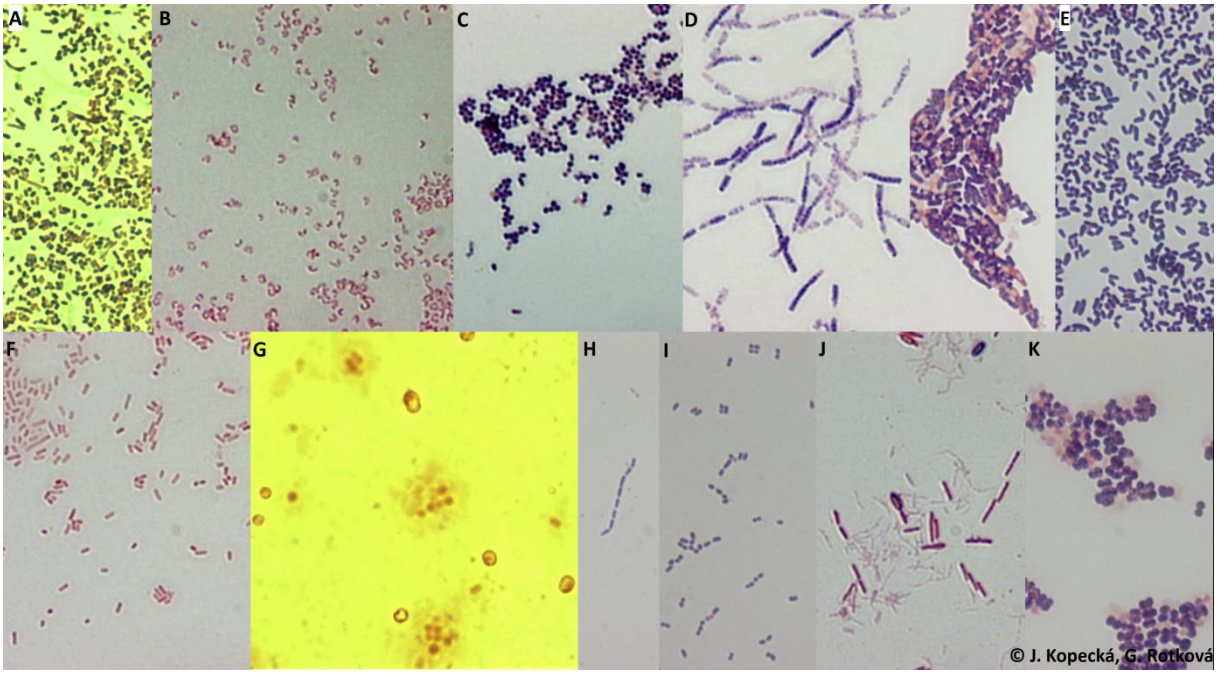

Fig. 66. Pure bacterial cultures stained according to Gram. *Acinetobacter* (A), *Arthrobacter* (B), *Bacillus mycoides* (C), *B. subtilis* (D), *Corynebacterium* (E), *Escherichia coli* (F), *Haloarcula hispanica* (G), *Lactobacillus casei* (H), *Leuconostoc mesenteroides* (I), *Paenibacillus polymyxa* (J), *Sporosarcina* (H).

Negative staining by nigrosin

- Transfer aseptically cells in drop of distilled water, add drop of nigrosin.
- Mix drops by loop and smear it by another glass (applied in angle of 45˚ all over the glass surface), let it dry on air.
- Observe at magnification 1000x with immersion oil.

Evaluation: Colourless cells on grey background are visible in preparation (fig. 67). Compare the shape and size of cells of various species of one bacterial genus. Results could be affected by thickness of dye layer (strong layer could be broken after drying) and concentration of dye.

Negative staining by Kongo red

- Transfer cells aseptically in drop of Kongo red.
- Smear it by another glaas (applied in angle of 45˚ all over the glass surface), let it dry on air.
- Pour it by 1 % of HCl, pour it immediately off, do not wash, dry it filter paper.
- Observe at magnification 1000x with immersion oil.

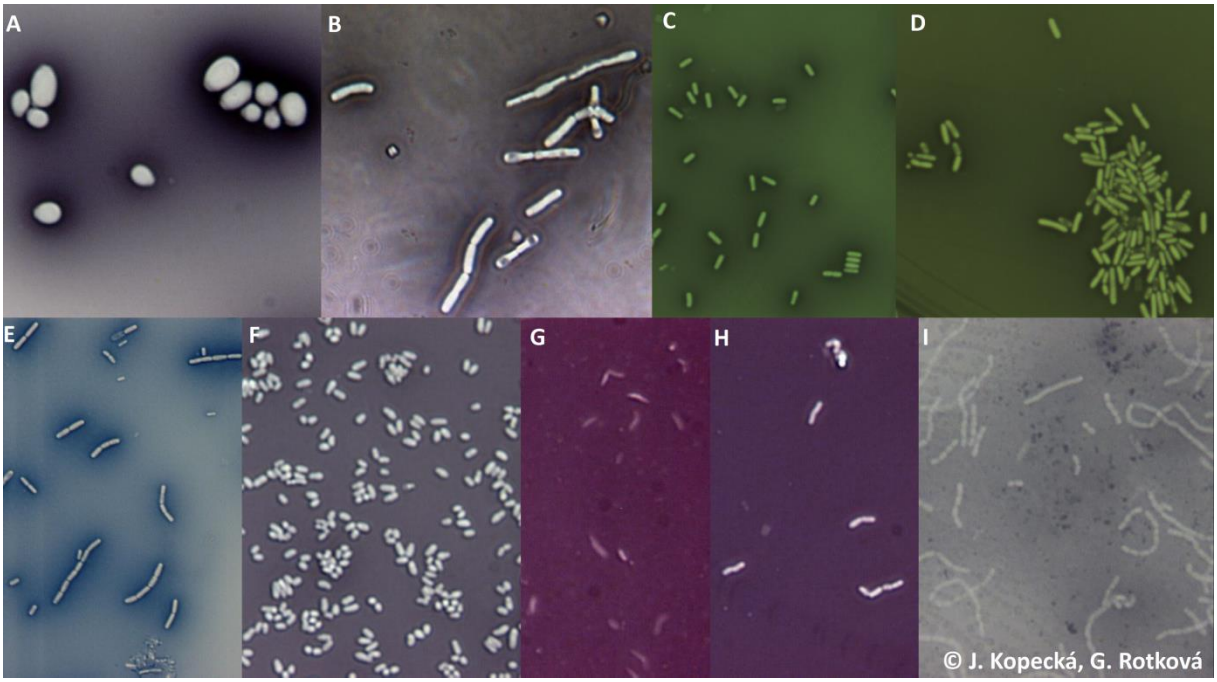

Fig. 67. Negative staining, *Saccharomyces cerevisiae* (A), *Bacillus cereus* (B), *B. subtilis* (C), *B. subtilis* (D), *Bacillus* sp. (E), *Corynebacterium* (F), *Haloferax mediterranei* (G), *Lactobacillus brevis* (H), *Lactobacillus casei* (I).

Native preparation for observing of shape and location of spores - genus *Bacillus* (phase and Nomarski contrast, fig. 68 a 69)

- Transfer cells in drop of sterile distilled water, mix it and cover it by cover glass (do not cover glass from upside, at first by one edge, do not push).
- Observe cells from liquid medium direct in medium without dilutin in drop of water.

- Observe immediately by phase or Nomarski contrast (objective 60x or 100x), preparattion is quicly drying.

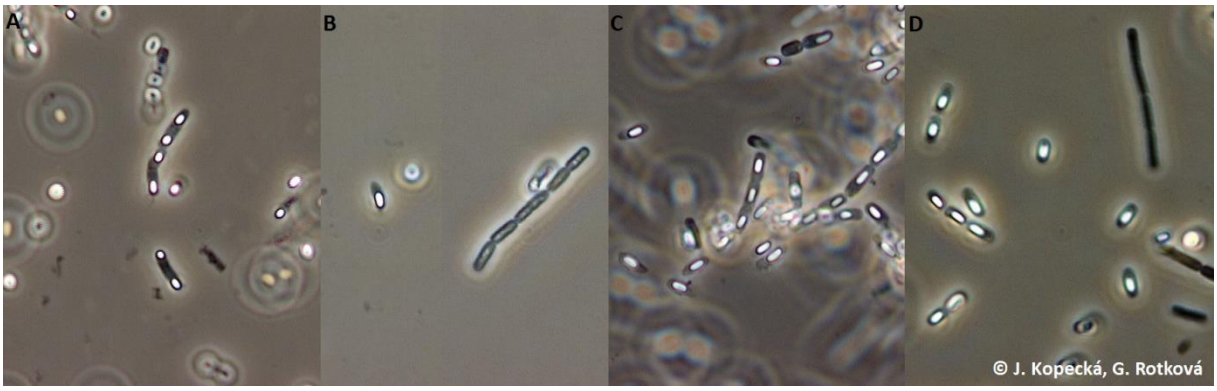

Fig. 68. Phase contrast in sporulating bacteria; *Bacillus megaterium* (A), *B. mycoides* (B), *B. pumilus* (C), *Lactobacillus brevis* (D).

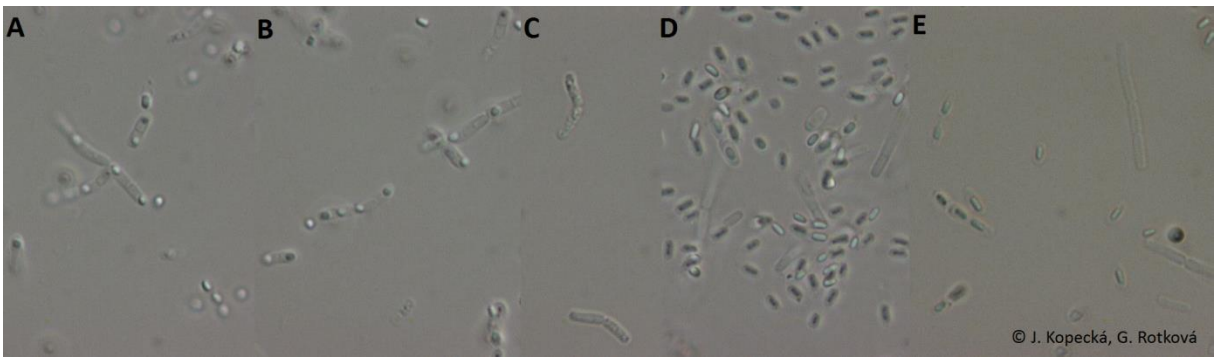

Fig. 69. Nomarski contrast in sporulating bacteria; *Bacillus megaterium* (A), *B. megaterium* (B), *B. mycoides* (C), *B. pumilus* (D), *Lactobacillus brevis* (E).

Conclusion

Compare the shape of cells, spores and ratio of lenght and width of cells. Is the bulge of cells cause by the spores? Is the bulge of cell central or terminal caused by spores? What affect the appearance of cells in preparate – age of culture, nutrient medium, magnification?

Additional information

Prescott L., M., Harley J. P., Klein D. A., Microbiology, WCB, Dubuque, 1996, ISBN 0-697-29390-4.

Sedláček I., Taxonomie prokaryot, Masarykova univerzita, Brno, 2007, ISBN 80-210-4207-9.

Control questions

1. Explain the terms bacterial species and bacterial culture.
2. Write at least 4 dyes used in microbiology.
3. Write at least 4 types of nutrient agar used in microbiology.
4. Complete the table:

Target structure	**Type of staining**
Cell wall	
Shape of cell, endospores, movement	
Real shape and size, capsule	
Capsule	
Spores	

Structures of cell (inclusion and capsule staining)

Aim of practice

Staining and observing of cell inclusions and capsules.

Introduction

Inclusion or inclusion bodies (granules of organic or inorganic compounds) are visible in light microscope and are located in cell matrix. Some of inclusions have not membranes and are presented in cytoplasm (polyphosphate and some glycogen granules), some inclusions are coated by 1-layer membrane (thickness of membrane is circa 2-4 nm), i.e. poly-β-hydroxybutyrate, some glycogen and sulphur granules, carboxyzomes, gas vesicules. Membranes of inclusions consist from proteins or lipids.

Phosphates are stored in form of **volutin** or **polyphosphate granules** and serve as source of phosphate, i.e. nucleid acid formation. Polyphosphate is linear polymer of orthophosphate linked by ester bonds. Polyphosphate serves as source of energy in some cases.

Fat is distributed in cell dispersed, in vacuoles or incorporated in membranes. Fat is accumulated in presence of oxygen, 2-3% of fat is commonly present in cell. Intracelular inclusions are founded especially in yeasts and micromycetes. Dye sudan III is mostly used for their staining, fat gets pink-red colour in 20 – 30 minutes (dye is solved in fat).

Glycogen is soluble polymer of glucose with α-1,4-bindings and with α-1,6-linking on every 8-10 monomer. It could form up to 50% of dry mass, is randomly located in cell matrix as small granules (20 – 100 nm). Glycogen is not visible in light microscope. Part of glycogen granules is coated by membrane. Glycogen is stained by Lugol solution. Bacterial glycogen is more linked. It serves as quick reserve, it is the primary storage polysaccharide in fungi. It serves as source of carbon for energy and biosynthesis.

Lugol solution composes from 1 % of iodine, 2 % of potassium iodide and distilled water. Potassium iodide is added to increase solubility of iodine in water. It is used as antiseptics, disinfection and as indicator of organic compounds presence, especially starch (change colour to blue-violet or black).

Some bacteria form layer of material that is located outside of cell wall. If this layer is well organised and do not wash out, it is called **capsule**. Capsule is composed from polysaccharides, but could contain another compounds. Capsules are visible in light microscope (negative staining). Capsule provides many advantages to bacteria: covers

pathogenic bacteria in macroorganism, avoids to drying due to higher content of water, keeps away many bacterial viruses and hydrophobic toxic compound, i.e. detergents.

Instruments and microorganisms

Saccharomyces cerevisiae

Bacillus cereus CCM 2010

Bacillus megaterium CCM 2007

Azotobacter

Leuconostoc

Methylene blue, Sudan III, Lugol solution

Kongo red, nigrosin

Microscopic slide, cover glass, loops, distilled water

Methods

Staining of volutin by polychromatic methylene blue (fig. 70)

- Dry the smear of the cells on air and cover it by polychromatic methylene blue (Löffler blue, maturated at least 12 months) for 1-3 minutes.
- Wash with water, dry it and observe at magnification 1000x.
- Purple volutin grains and light blue cytoplasm are visible in preparation.

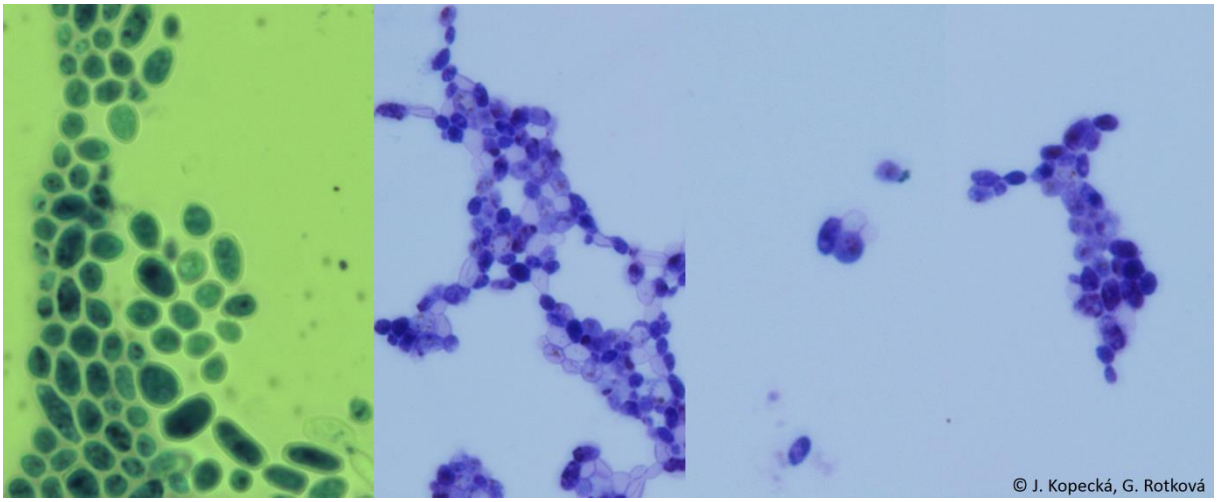

Fig. 70. Volutin in *Saccharomyces cerevisiae*.

Fat – staining by Sudan III (fig. 71)

- Mix cells with dye Sudan III for 10 - 30 minutes.
- Cover it by cover glass and observe brick-like red drops of fat.

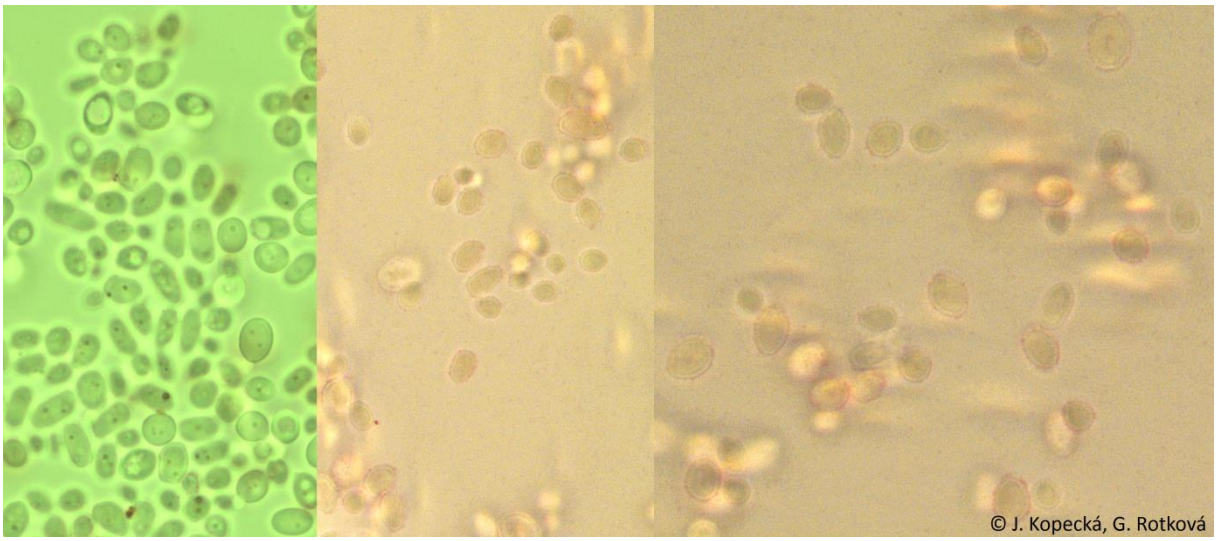

Fig. 71. Fat in *Saccharomyces cerevisiae*.

Glycogen (fig. 72)

- Prepare native preparation, drop the Lugol solution beside the cover glass.
- Observe immediately brown granules of glycogen.

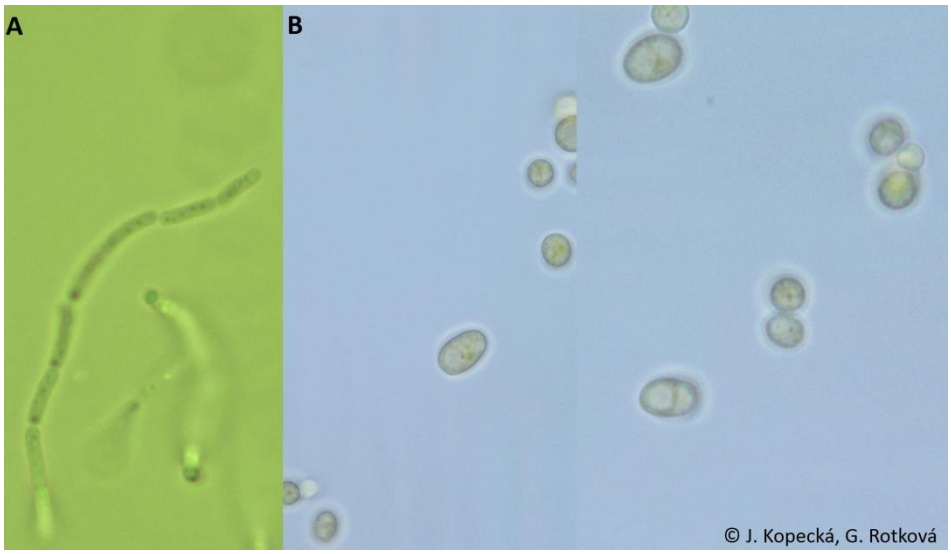

Fig. 72. Glycogen in *Bacillus megaterium* (A) and *Saccharomyces cerevisiae* (B).

Negative staining of capsules by nigrosin - genus *Azotobacter*

- Transfer small amount of cells from mucilaginous colony in drop of water, mix it with drop of nigrosin and cover it by cover glass. Drain the rest of liquid by filter paper and observe.
- Grey cells are surrounded by white capsules, background is dark. Nigrosin stains cells and background, not capsules.

Negative staining by nigrosin, afterstaining of cells by methylene blue - genus *Azotobacter*

- Mix the drop of nigrosin with drop of water, transfer cells in suspension, smear it over the glass and let it dry.
- Cover the smearing by methylene blue solution for 3 minutes, wash it with water and dry.
- Observe at magnification 1000x with immersion oil.

Evaluation: Blue cells are surrounded by light capsules, background is dark.

Negative staining of capsules by Kongo red, afterstaining of cells by methylene blue – genus *Azotobacter*

- Transfer cells in drop of Kongo red, smear suspension over glass and let it dry.
- Cover the smearing by HCl for several seconds. Pour off the acid, do not wash, dry it by filter paper.
- Cover it by methylene blue for 3 minutes, pour it off, wash it with water and dry it on air.
- Observe at magnification 1000x with immersion oil.

Evaluation (fig. 73): Blue cells and cysts surrounded by light capsules on dark background.

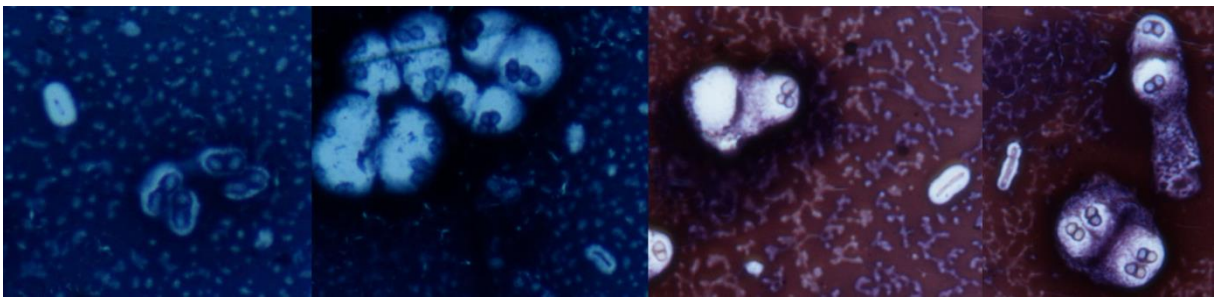

Fig. 73. Negative staining of genus *Azotobacter*.

Conclusion

Was the inclusions stained? Were the capsula and cells highlighted by the negative staining?

Additional information

Prescott L., M., Harley J. P., Klein D. A., Microbiology, WCB, Dubuque, 1996, ISBN 0-697-29390-4.

Control questions

1. What are inclusions?
2. Is every inclusion covered by the membrane?
3. As source of what shloud be used the volutin granules by the cell?
4. As source of what shloud be used the glycogen by the cell?
5. What is the advantage of presence the capsule for pathogennic bacteria?
6. Describe the structure of bacterial capsule?

Movement of cells

Aim of practice

Observing of cell movement microscopically and macroscopic observing of bacterial motion on Petri dish. Observing of flagellar motion in separated drop. Staining of flagella and their observing in brief filed and phase contrast. Innoculation of semifluid medium – in Petri dishes and in tubes.

Introduction

Most of moving bacteria moves due to the flagella that is anchored in outer part of plasma membrane and in cell wall. Flagellum has circa 20 nm in diameter and is 15 – 20 µm long. Flagellum is visible after staining in microscope, staining increases its thickness. Location of flagellum is various (fig. 74).

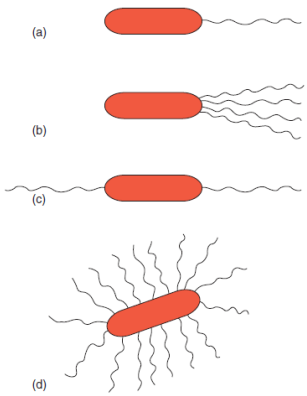

Fig. 74. Arrangement of flagella on bacterial cells. (a) In monotrichous, polar, a single flagellum is located at one end of the cell; (b) in lophotrochous, polar, many flagella are grouped at one end of the cell; (c) in amphitrichous, polar, a single flagellum is located at both ends of the cell; (d) in peritrichous, flagella are located all around the cell (Prescott et al. 1996, adjusted).

Bacterial flagellum comprises from three basic parts. The longest part is filament, basal body is anchored in cell linked through hook. Filament is hollow and robust, composed from one protein, flagellin. Differences in flagellum composition in gramnegative and grampositive bacteria are shown in fig. 75.

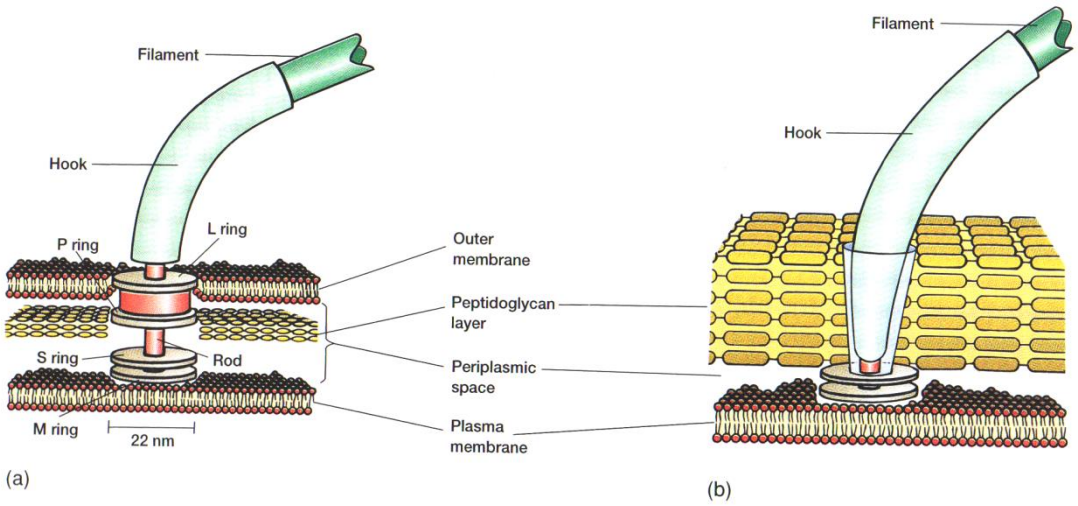

Fig. 75. The ultrastructure of bacterial flagella. Flagellar basal bodies and hooks in (a) gramnegative and (b) grampositive bacteria (Prescott et al. 1996, adjusted).

Direction of flagellar rotation determinates the nature of bacterial movement (fig. 76). Bacteria move natural by Brown motion except for flagellar motion. Spirochetes and helical bacteria could move due to mucous, s.c. axial filament. Another movement is gliding motility in cyanobacteria and some genera of myxobacteria and mycoplasma.

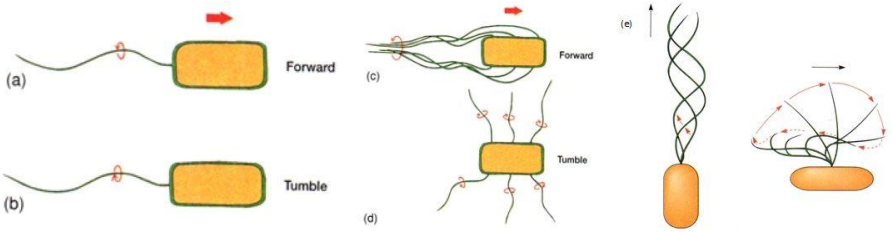

Fig 76. Flagellar motility. The relationship of flagellar rotation to bacterial movement. Parts (a) and (b) describe the motion of monotrichous, polar bacteria. Parts (c) and (d) illustrate the movements of peritrichous organisms and part (e) describe motion of polar flagella (Prescott et al. 1996, adjusted).

Characteristics of some microorganisms

Bacillus – straight rods in pairs or chains; grampositive; peritrichous; endospores formation

Pseudomonas – gramnegative; straight or curved rods; one or some polar flagella

Proteus – gramnegative; straight rods; peritrichous

E. coli – gramnegative; single cells, pairs; peritrichous

Instruments and microorganisms

young cultures (4 or 16 hours old)

Bacillus cereus CCM 2010

Pseudomonas fluorescens CCM 2115^{T}

Proteus vulgaris CCM 1799

Escherichia coli CCM 3954

Micrococcus luteus CCM 169 – for comparing of native preparation, immobile

Methods

Glass aids shloud not be used for preparation flagella preparations (except for microscopic slide) because of easy flagella break off. It is necessary work with young cultures (4-16 hours old), old cultere release often flagella.

Observing of flagella motion - separated drop

- Prepare native preparation, pipette drop of suspension on microscopic slide.
- Do not cover by cover glass, observe by objective 20x by Nomarski contrast, do not immerse!
- Sufficient amount of oxygen is important for cell vitality, more sensitive cells are moving unly in the upper layer of drop.
- It is necessary to focus carefully on the upper part of drop.

Staining of flagella (fig. 77)

Dye contains disinfectant/mordant tanin that coats over the flagellum, increases its diameter and makes it visible.

- Prepare native preparation and cover it carefully by cover glass.
- Drop dye for staining of flagella beside the cover glass.
- Let the dye absorb through the preparation by filter paper and observe preparation at magnification 1000x with immersion oil.

Dye for staining of flagella: solution I (10 pieces) and II (1 piece), store at -20 °C.

Solution I: 10 ml of 5% water solution of phenol, 2 g of tannin, 10 ml of Kal $(SO_4)_2$ * 12 H_2O

Solution II: saturated solution of crystal violet (12 g) in ethanol (10 ml, 96%)

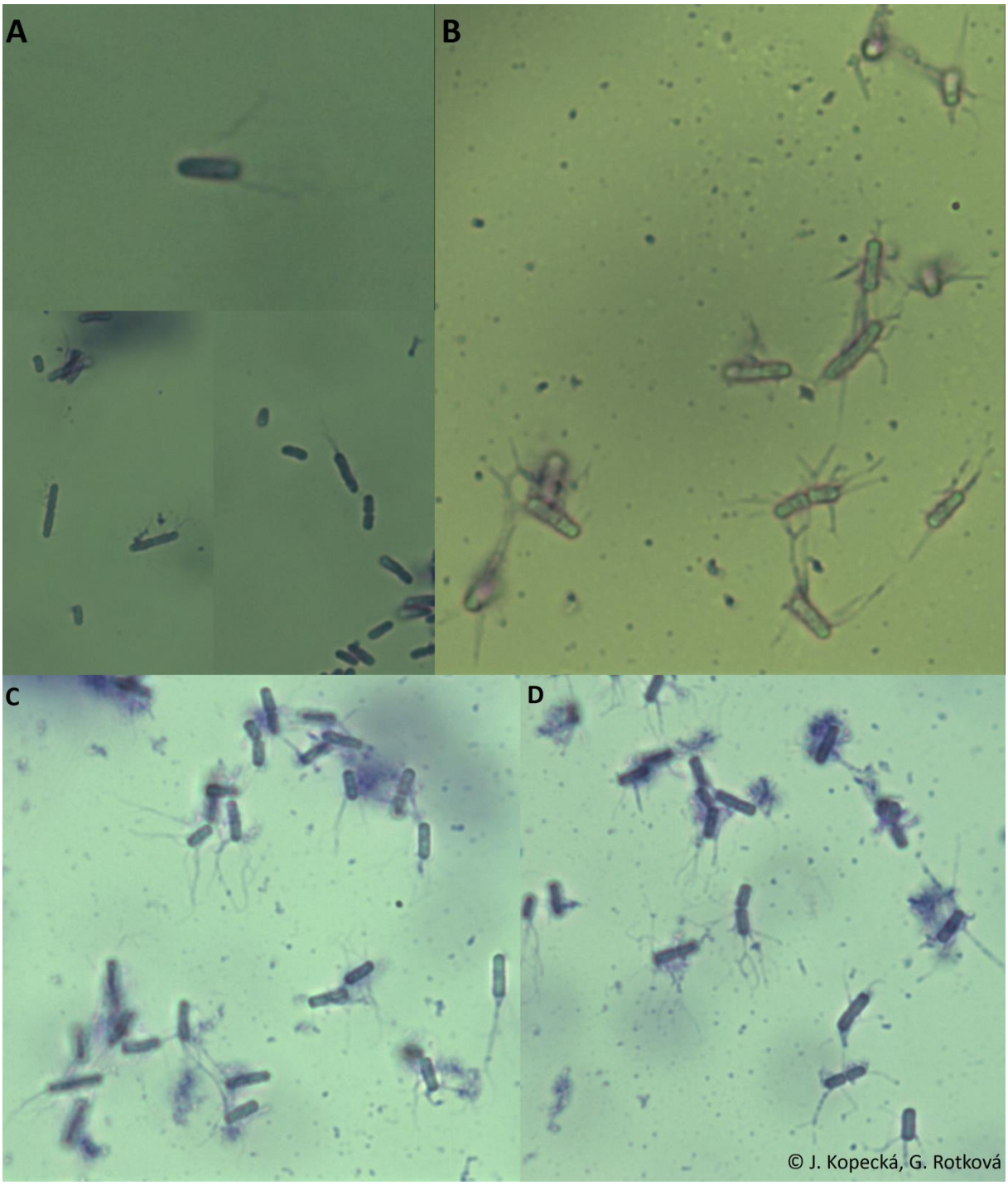

Fig. 77. Stained flagella of *Proteus vulgaris* (A) and *Bacillus cereus* (B, C, D).

Obseving of motion on agar for movement testing

Medium contains low amount of agar, semi-liquid medium (lower viscosity of environment).

- Innoculate agar by one stab in the middle of agar to grow only 1 colony.
- Stab the loop with culture in the middle of dish.
- Moving cultures form spreading circle, sometimes with s.c. waves. Non-moving/Immobile cultures grow only in place of stab to certain size of colony.

- Results are visible in 3-5 days. Do not move with dishes.

Medium content: 100 ml of demineralization water, 0,1 g of yeast extract, 0,01 g of K_2HPO_3, 0,2 g of agar.

Solid medium (phenomenon of swarming motility) and semi-solid medium (floating cells in medium) can be used for genus *Proteus*.

Content of Luria-Bertani swarming medium with 0,7 % agar: 100 ml of distilled water, 1 g of enzymatic hydrolysed casein, 0,5 g of yeast extract, 1 g of NaCl, 0,7 g of agar.

Conclusion

Was the movement of bacteria good observable in suspended drop? Were the flagella stained by tannin? Were the differences remarkable between monotrichous and peritrichous bacteria? Was the motion of bacteria observable on the dish (massive growing colonies)?

Additional information

Prescott L., M., Harley J. P., Klein D. A., Microbiology, WCB, Dubuque, 1996, ISBN 0-697-29390-4.

Sedláček I., Taxonomie prokaryot, Masarykova univerzita, Brno, 2007, ISBN 80-210-4207-9.

Control questions

1. Describe types of motion: Brown motion, movement by flagella and gliding motility.
2. Write the differences in flagella structure between grampositive and gramnegative bacteria.
3. What protein comprises the bacterial flagella?
4. What is used for highlighting of bacterial flagella for light microscopy?
5. Why are not used the glass aids during the preparation and observing of flagella?

Acid-fast staining

Aim of practice

Acidoresistant (acid-fast) staining and Gram staining of gramvariable cells.

Introduction

Gramvariable or gramlabile microorganisms contain part of cells that is stained as grampositive and part of cells that is stained as gramnegative in pure culture.

Acid-fast microorganisms contain high level of glycolipids, especially mycolic acids, fat acids and higher alcohols in cell wall. They do not colour by inorganic acids or by mixture acid and alcohol according toZiehl-Neelsen staining. Acidoresistance is important for distinguishing of some bacterial species, i.e. *Mycobacterium tuberculosis* and *M. leprae*. Acid-fast bacteria are relatively impermeable and resistant to weak dyes. If they are stained by strong dye (i.e. basic fuchsin soluble in 5 % of phenol), they do not colour not even by 20 % sulphuric acid. Acidoresistance depends on cell integrity, lipid content and their special anatomic composition.

Characteristics of some microorganisms

***Acinetobacter* –** short rods in pairs or chains; gramnegative or gramvariable (fig. 78)

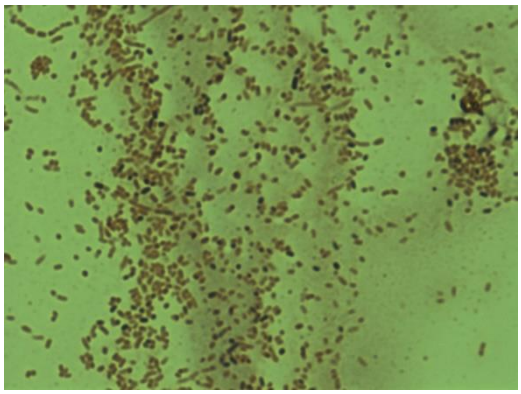

Fig. 78. *Acinetobacter grimontii* CCM 7198^{T} stained according to Gram method.

Corynebacterium – straight or curved rods with narrowed ends or short palisades; grampositive; non-acid-fast

Mycobacterium – straight or curved rods, sometimes linked; do not form aerial hyphae or capsules; week grampositive; acid-fast; some species grow very slowly (months)

Nocardia – branched filaments of substrate mycelium growing on surface or in agar, aerial mycelium formation; grampositive or gramvariable; some species are acid-fast

Rhodococcus – rods, branched substrate mycelium, filaments are falling into short rods and cocci; some species produce week aerial haphae; grampositive, could be acid-fast

Instruments and microorganisms

Mycobacterium phlei CCM 5639 – acid-fast

Nocardia carnea CCM 2756 – acid-fast

Rhodococcus erythropolis CCM 277

Corynebacterium glutamicum CCM 2428

Acinetobacter grimontii CCM 7198^{T}

Methods

Gram staining

- Drop sterile distilled water on microscopic slide.
- Transfer little amount of cells in drop of water.
- Smear the suspension over the glass, let it dry and fix by the flame (pull the glass through flame three times).
- Put the glass in solution of crystal violet for 30 seconds, wash it with water.
- Put the glass in Lugolova solution for 30 seconds, wash it with water.
- Cover the glass by ethanol/acetone, for maximum 15-20 seconds, wash it with water.
- Put the glass in safranin for1 minute, dry it by filter paper and observe at magnification 1000x.

Acid-fast staining according to Ziehl-Neelsen

- Prepare flame-fixed preparation.
- Cover it by concentrated carbolfuchsin, warm it to evaporation. If it is necessary, supply the dye. Warm the preparation for 3-5 minutes (do not boil the preparation).
- Wash by acidic alcohole two times for maximum 15 seconds.
- Stain by Löffler methylene blue or malachite green for 30 seconds.
- Wash with water and observe at magnification 1000x.
- Red coloured acid-fast cells should be good differentiated compared with green or blue background (fig. 79).

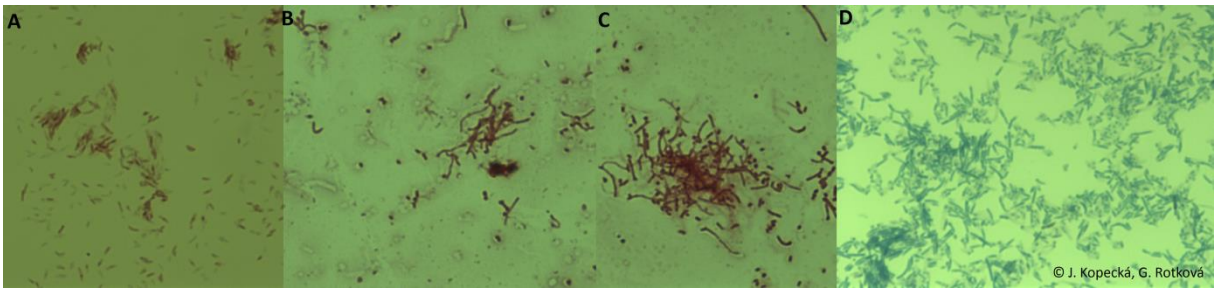

Fig. 79. Acid-fast staining according to Ziehl-Neelsen, *Mycobacterium phlei* (A), *Nocardia carnea* (B, C), *Rhodococcus erythropolis* (D).

Conclusion

Were the acid-fast cultures stained by the Gram staining? Did the acid-fast culture decolorize by the acidic alcohol? What colour had the cells after acid-fast staining?

Additional information

Greenwood D., Slack R. C. B, Peuthere J. F., a kol., Lékařská mikrobiologie, Grada Publishing, 1999, ISBN 80-7169-365-0.

Sedláček I., Taxonomie prokaryot, Masarykova univerzita, Brno, 2007, ISBN 80-210-4207-9.

Control questions

1. What is the aim of acid-fast staining?
2. What is the reason of post-dying with methylene blue in acid-fast staining?
3. Acid-fast staining is used for pathogenic agent, which disease?
4. What is the reason of microbial acidoresistance?

Glass cultures

Aim of practice

Cultivation of actinomycetes. Observing of aerial, substrate mycelia and comparing the differences.

Introduction

Actinomycetes (i.e. *Actinomyces*, *Nocardia*, *Rhodococcus*, *Streptomyces*) are grampositive bacteria (prokaryotic cell structure) with high amount of G+C content, form mycelium, reproduce by spores. Mycelium (stabile or temporary) is the complex of hyphae with net structure. Yeast could form s.c. pseudomycelium. Yeast cells bud on poles and single cells are nt separated from each other and stay connected. Filament of pseudomycelium is composed from elongated cells and looks like strangled. Hyphae of mycelium are separated by septa, have the same diameter over the whole lenght. **Substrate mycelium** grows in to the substrate, **aerial mycelium** has parts over the substrate (fig. 80). Aerial mycelium produce single spores, pairs of spores, chains or various vesicles of spores, s.c. sporangium or sporophore. Sporangia could be located on substrate mycelium too, if aerial mycelium is not formed. Shape and location of spores or sporangia is significant identification marker. Nocardioform actinomycetes form only substrate mycelium (*Rhodococcus*), substrate and aerial mycelium (*Nocardia*) or only aerial mycelium (*Sporichthya*). Streptomycetes form extensively branched, long filaments, substrate and aerial mycelia with round spores in chains. Size of cell is circa 0,5-2,0 µm. They grow slowly, colonies are formed after 3 - 4 days. Matured aerial mycelium is appeared after 7 - 14 days. Hyphae are white, but they are coloured after some time. Actinomycetes could grow in liquid medium, but demand intensively aeration and shaking/stirring because of cluster formation. They are widely spreaded in nature, soil, on plants; they could be pathogenic for humans, animals and plants. Nocardioform actinomycetes and streptomycetes form characteristic colonies with rough, wrinkled surface and pigmentation.

Microscopic preparation mostly damages intact structure of substrate and aerial mycelium by taking of sample. Glass culture facilitates to observe hyphae and filament direct growing on cover glass. Innoculation of glass cultures have to be strictly aseptic to avoid contamination, preferably in flowbox, by sterile loop, scalpel and pincette. Medium is choosen according to microorganism.

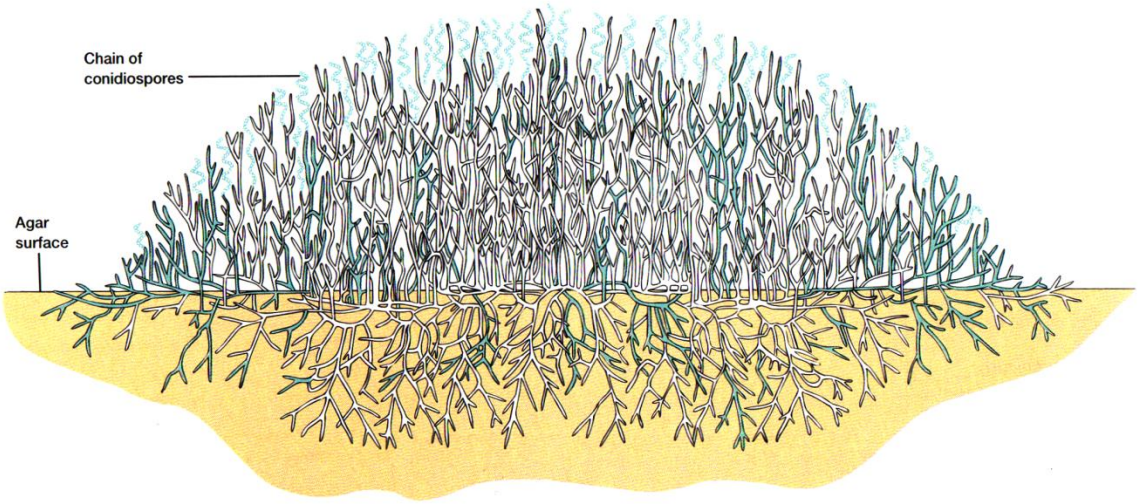

Fig. 80. An actinomycete colony, the cross section of colony with living (colored) and dead (white) hyphae. The substrate mycelium and aerial mycelium with chains of conidiospores are shown (Prescott et al. 1996, adjusted).

Characteristics of some microorganisms

Nocardia – moderately or extensively branched filaments of substrate mycelium growing on surface or in agar, aerial mycelium formation; grampositive or gramvariable; some strains are acid-fast

Rhodococcus – rods, extensively branched substrate mycelium, filament sis separated in short rods and cocci; some strains produce weak aerial hyphae; grampositive, could be acid-fast

Streptomyces – vegetative filaments form branched mycelium; aerial mycelium forms chains of spores; pigments production; filament formation; colonies with appearance of lichen, leather

Instruments and microorganisms

Sterile pincette, scalpel, distilled water, cover glass

Sterile dish with glass beads and microscopic slide

Nutrient media for target cultures (thick layer for cultivation on cover glass, thinn layer for cultivation on microscopic slide)

Innoculation loop

Rhodococcus erythropolis CCM 277 (fig. 81)

Nocardia carnea CCM 2756 (fig. 82)

Streptomyces griseus ssp. *griseus* CCM 3362 (fig. 83)

Fig. 81. Substrate mycelium of *Rhodococcus erythropolis.*

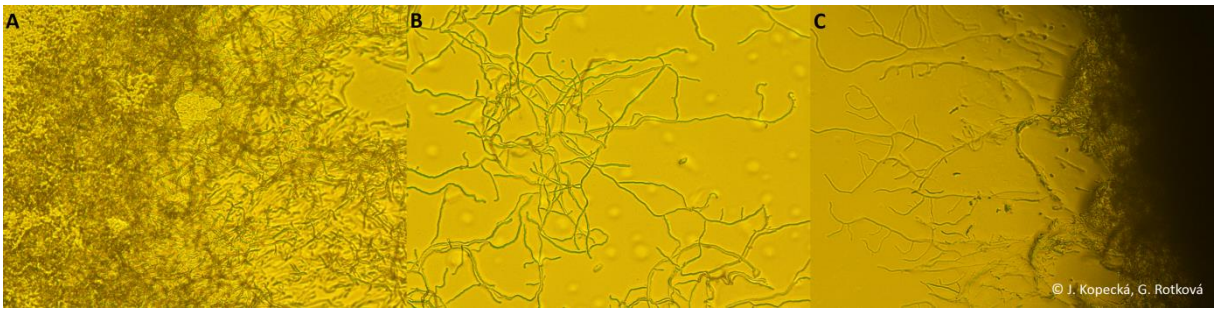

Fig. 82. *Nocardia carnea*, substrate (A), aerial (B) mycelium and the croos between them (C).

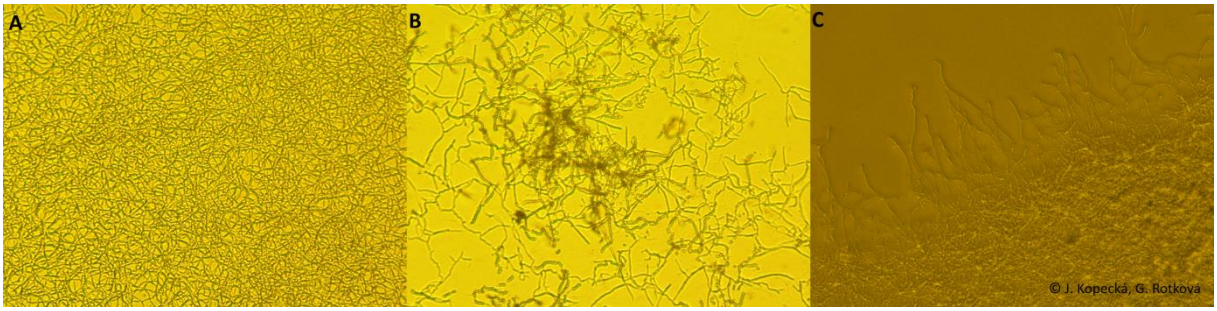

Fig. 83. *Streptomyces griseus*, substrate (A), aerial (B) mycelium and the croos between them (C).

Methods

Wet chambers – cultivation on microscopic slide (fig. 84)

- Place the microscopic slide aseptically on glass beads in sterile Petri dishes.
- Place the thin square of agar on microscopis slide by sterilised scalpel.
- Choose the type of agar according to microorganism: *Nocardia* - medium M8; *Rhodococcus* - medium M2 or M8; *Streptomyces* - medium M15.
- Innoculate edges of agar and cover it by sterile cover glass (18x18 mm or 22x22 mm).
- Pipette circa 5 ml sterile distilled water on glass beads under microscopic slide.

- Cultivaton is performed at 30 °C for 3 – 5 days.

Observing: direct, growth on glass, magnification 200x nebo 400x, Nomarski contrast.

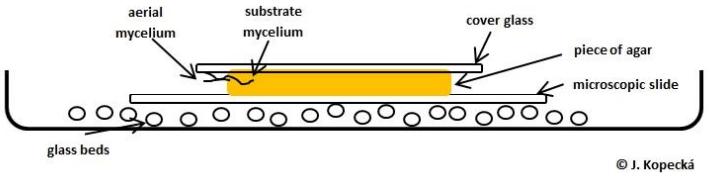

Fig. 84. Cultivation on microscopic slide.

Cultivation on cover glass (fig. 85)

- Use medium according to strain – thick layer of agar on Petri dish.
- Stab the sterile cover glass in agar by sterile pincette in angle 45 °.
- Innoculate culture from upper side of cover glass. Substrate mycelium will grow through agar.
- Pull out the glass before observation (after 3-5 days) and sign the upper and lower side for recognising of aerial and substrate mycelium.
- Observing: Put the cover glass with growed culture to microscopic slide. Observe at magnification 400x in brief filed or by Nomarski contrast.

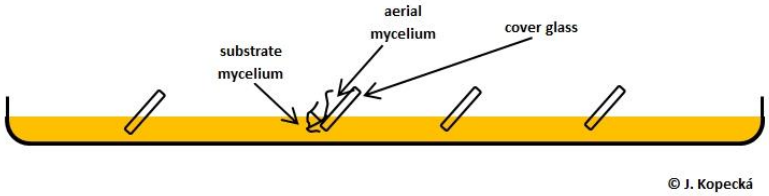

Fig. 85. Cultivation on cover glass.

Conclusion

Did you succeed in innoculation of glass cultures? Was the difference evident between substrate and aerial mycelia? What was the type of mycelia, size and location of sporangia, if they were present?

Additional information

Prescott L., M., Harley J. P., Klein D. A., Microbiology, WCB, Dubuque, 1996, ISBN 0-697-29390-4.

Sedláček I., Taxonomie prokaryot, Masarykova univerzita, Brno, 2007, ISBN 80-210-4207-9.

Control questions

1. Which bacteria and yeasts are cultivated by glass culture technique and why?
2. Which nutrient media are used in this practice?
3. Why is important the aseptic work?
4. What is the mycelium, which microorganisms form mycelium and what types of mycelia do we recognise?
5. What is the pseudomycelium?

Fluorescence

Aim of practice

Fluorescence microscopy. Observing of autofluorescence and flourescence of microorganisms caused by the fluorochrome (dye) at different wave-lenghts.

Introduction

Fluorescence is the ability of some compounds to emit light with longer wave-lenght after radiation with shorter wave-length (excitation radiation). Two types of fluorescence could be distinguished, primary (autofluorescence) and secondary. Autofluorescence is the ability of some natural compounds (i.e. chlorophyll) fluoresce after exposition to UV radiation. Secondary fluorescence is the fluorescence of dye s.c. fluorochrome that is covalent binded to target cell structure. Fluorochromes are i.e. fluorescein, rhodamin, auramin and acridin orange.

SYBR Green dye binds nucleid acids (the highest afinity to dsDNA). Complex nucleid acid-SYBR Green absorbs blue light (λmax = 497 nm) and emits green light (λmax = 520 nm).

Sample is exposed to ultraviolet, violet or blue light during the fluorescent microscopy. Microscope have to be equipped with source of short-wave-length light (xenon discharge lamp). Light is going through two set of light filters. First set (excitation) transmits only target wave-lengths and eliminates rays with longer wave-length. Excitation filters are glass with various thickness, blue or violet. Seconds set eliminates the entry of short-wave-length to human eye by elimination filter. Condenzor with dark field provides black background on that fluorescent objects shine.

Pseudomonas fluorescens – produce fluorescein; occurence in soil, water, food, human clinic material

Instruments and microorganisms

Saccharomyces cerevisiae

Bacillus sp.

Pseudomonas fluorescens

MPA, SYBR Green, PBS buffer

Methods

Autofluorescence (fig. 86)

- Expose the growth of fluorescent culture to UV radiation.

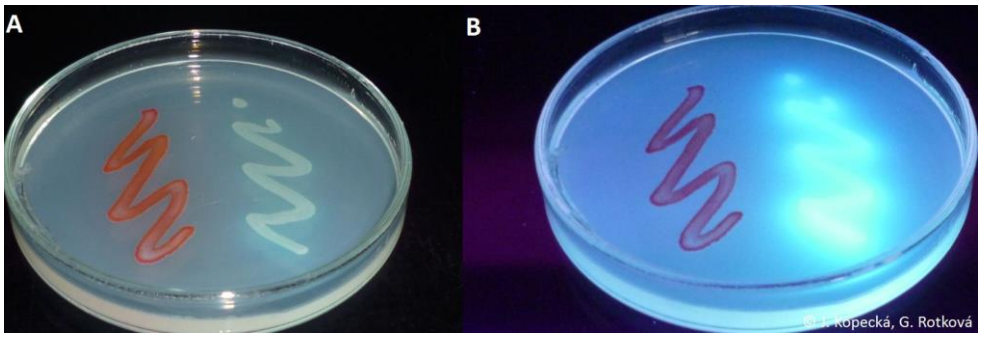

Fig. 86. Culture of *Serratia marcescens* (red pigment) and *Pseudomonas fluorescens* exposed to light (A) and to UV radiation (B).

Fluorescence emited by SYBR Green dye (fig. 87 and 88)

- Wash the culture in PBS buffer, dye it by SYBR Green dye and observe by fluorescent microscope at various wave-lenghts.

PBS (phosphate buffered saline): 1,81 g of $Na_2HPO_4{*}12H_2O$, 0,203 g of $NaH_2PO_4{*}2H_2O$, 0,85 g of NaCl, 100 ml of H_2O, pH 7

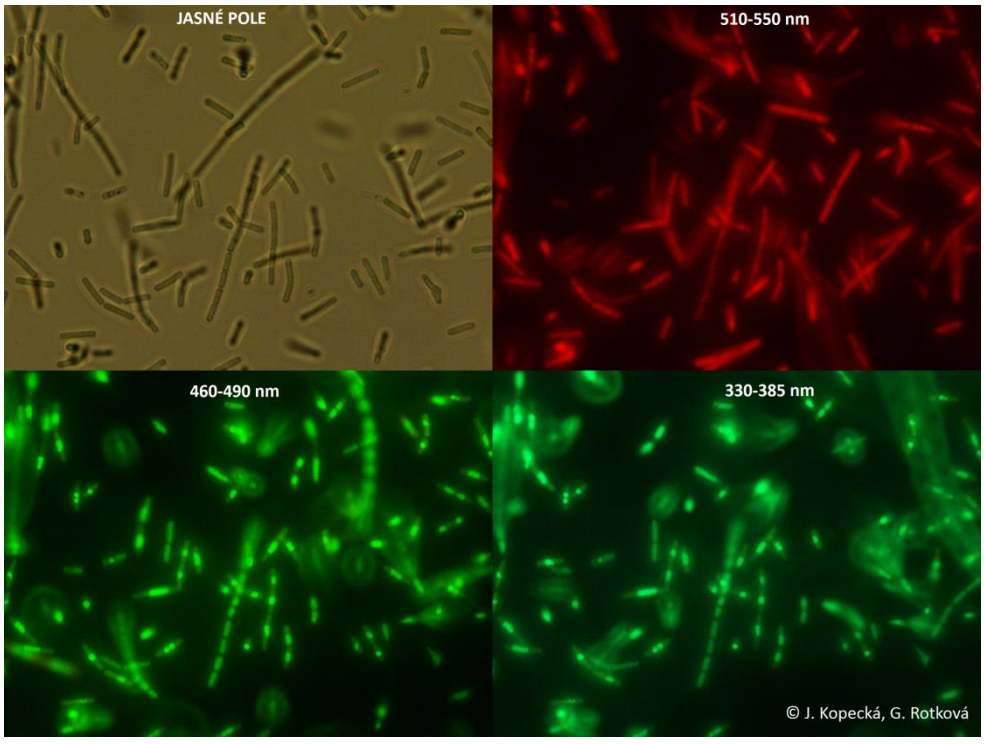

Fig. 87. Culture of *Bacillus thuringiensis* stained with SYBR Green dye and exposed to the radiation with different wave-length (jasné pole = brief field).

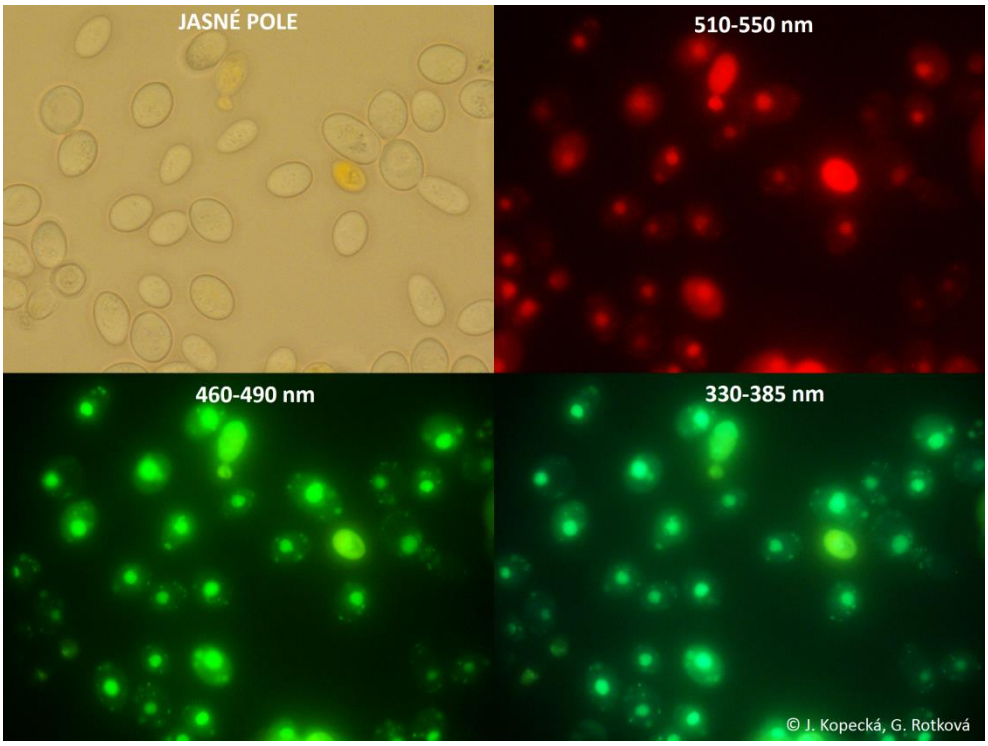

Fig. 88. Culture of *Saccharomyces cerevisiae* stained with SYBR Green dye and exposed to the different wave-length (jasné pole = brief field).

Conclusion

Did the pure culture fluoresce at UV radiation (dish/microscope)? Were the differences of fluorescence evident at various wave-length, why?

Additional information

Prescott L., M., Harley J. P., Klein D. A., Microbiology, WCB, Dubuque, 1996, ISBN 0-697-29390-4.

Sedláček I., Taxonomie prokaryot, Masarykova univerzita, Brno, 2007, ISBN 80-210-4207-9.

Votava M., Lékařská mikrobiologie – vyšetřovací metody, Brno, Neptun, 2010, ISBN 978-80-86850-04-8.

Control questions

1. What part of cell show fluorescence after SYBR Green dye binding?
2. What causes the autofluorescence?
3. On what is binding SYBR Green dye?