University of Szeged

Advanced Microbiology Practicals

for MSc students

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2019

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Description of the subject

Master level

(1.) Title of the subject: Advanced microbiology practicals 1 and 2	**Credits:** 6/6
Type of the subject: compulsory	
The ratio of the theoretical and practical character of the subject: 10-90 (credit%)	
The type of the course: laboratory practice **The total number of the contact hours**: 4 per week **Language***: English*	
Type of the evaluation: written test **Other methods for evaluation of the student's competence:** preparation of a note-book, skills during the practice	
The term of the course: II-III. semester	
Prerequisite of the subject: Advanced microbiology I.	

Description of the subject

The aim of the subject:
The courses are directed to students who have some skills in basic microbial techniques. It provides an overview of techniques generally used in environmental and molecular microbiology; methods used in microbial ecology are also presented. Participants will acquire techniques generally used to manipulate microorganisms and they will study the genetic background of the life cycle of two important eukariotic modell systems *Aspergillus nidulans* and *Saccharomyces cerevisiae*.

Course description:

- The sexual life cycle of *Aspergillus nidulans* (heterokaryon formation, diploid isolation, ascospore formation and genetic analysis of the progenies)
- The parasexual life cycle in filamentous fungi (heterokaryon formation by anastomosis and protoplast fusion, diploid isolation, haploidization);
- Study of the sexual life cycle of *Saccharomyces cerevisiae* (conjugation, diploid isolation, spore forming, random spore analysis);
- The killer phenomenon in yeasts;
- The presence of dsRNA viruses in *Saccharomyces cerevisiae*;
- Counting of viable bacterial and fungal cells in soil samples;
- Counting of psychro-, meso- and thermophilic bacteria in water samples;
- Selective isolation of *Pseudomonas* and *Bacillus* strains from soil;
- Selective isolation of Trichoderma strains from environmental samples;
- In vitro testing of the microbial antagonism between bacteria and filamentous fungi;
- Study of the extracellular lipase and esterase secretion of bacterial strains;
- Detection and measurement of the cellulase, xylanase and phosphatase activity of *Trichoderma* strains;
- Study of the extracellular amylase, chitinase and protease production of bacterial strains;
- Isolation of laccase-producing filamentous fungi;
- Determination of the copper tolerance of bacteria and fungi;

- Determination of the susceptibility of *Trichoderma* strains to fungicides.

Selected bibliography (**2-5)** (author, title, edition, ISBN)

Baltz, R. H., Davies, J. E., Demian, A. L. (2010): *Manual of Industrial Microbiology and Biotechnology.* Third Edition. ASM Press, Washington DC.
Lammert J.M.: Techniques in Microbiology: A Student Handbook 1st Edition, ISBN-13: 978-0132240116
HERSKOWITZ I.: Life cycle of the Budding Yeast *Saccharomyces cerevisiae*. MICROBIOLOGICAL REVIEWS, 1988, 536-553, 0146-0749/88/04536-18$02.00/0
Pontecorvo G (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5: 141–238.
Wickner R.B., Fujimura T., Esteban R.: Viruses and prions of *Saccharomyces cerevisiae*. Adv Virus Res. 2013; 86: 1–36. doi: 10.1016/B978-0-12-394315-6.00001-5
Hamari Zs., Pfeiffer I., Takó M., Vágvölgyi Cs.: Advanced microbiology practicals (typescript)

General competence *(knowledge, skills,* etc., *KKK **8.***) **promoted by the subject**

a) knowledge

- Familiar with the tools and methods applied in modern biology.
- Know the terminology of microbiology and apply it in the correct way.
- Understand the social problems with biological relevance.
- Understand the significance of the interdisciplinary approach.
- Know the coherency among the area of biology.

b) skills

- Able to participate in biological research project and to create new scientific results under competent supervision.
- Able to plan research projects in the field of biology
- Able to recognize the coherency among the different area of biology.
- Able to apply new tools and techniques independently.
- Able for the interpretation and the presentation of the results.

c) attitude

- open to cooperate with other research groups
- ready to understand the evolution, structure and function of the living organisms
- interested in new results, techniques and methods; contribute to new scientific results and methods
- keep the ethical rules of the biological research

d) autonomy and responsibility

- able to organize the work of small research teams independently
- provide and require safety work conditions in the laboratory
- help his collegues in the completion of the research projects
- build his/her scientific career consciously

Special competence promoted by the subject:

Knowledge	Skills	Attitude	Autonomy/responsibility
Know the way how to carry out	Able to carry out sterile work in	Students are committed to the	Always observe the laboratory rules.

experiments to study the sexual and parasexual life cycles of eukaryotic microorganisms	microbiological laboratory.	sterile work in the microbiology lab and always keep the laboratory rules working with living materials. Open to study new techniques and methods. Ready to prepare a correct and up-to-date notebook. Ready to cooperate with his/her colleagues in the completion of the experiments.	Apply the acquired methods independently. Evaluate the results independently.
Know the method for genetic analysis of the progeny	Students can apply protoplast fusion and sexual cross to obtain heterokaryon.		
Know the methods to isolate microbes from different environmental samples.	Able to apply the media and methods for isolation of microbes from the environment.		
Know the counting methods for evaluation the microbial cell numbers in environmental samples.	Plan experiments to count the cell number in environmental samples.		
Acquire techniques to detect and measure extracellular enzyme activity of microorganisms.	Present that microbes produce different kind of extracellular enzymes.		
Know methods to detect antagonistic relationships among microbes.	Demonstrate the antagonistic relationship between the microbes.		
Carry out susceptibility tests.	Able to plan experiments to reveal the drug susceptibility of microbes.		
List the types of killer toxins and their chemical composition.	Able to demonstrate that killer toxins have different chemicals composition.		
Know the genetic background of killer toxin production.	Able to carry out experiment to reveal the genetic background of		

	killer toxin production.		

Instructor of the course (*name, position, scientific degree*)**:**
Papp Tamás associate professor PhD

Teachers (*name, position, scientific degree*)**:**
Hamari Zsuzsanna associate professor PhD
Pfeiffer Ilona associate professor PhD
Takó Miklós senior lecturer PhD

1. How to work in a microbiological laboratory: instructions and rules

1. General Introduction

The purpose of this practical handbook is to support the preparation for microbiological practices, furthermore, with the detailed description of the experiments it serves as a guide for students in the laboratory. Experiments could be performed independently; at the same time, they are didactically intertwined, and they relate to the knowledge acquired in theoretical courses.

At the beginning of the handbook, the microbiological laboratory safety regulations are briefly described followed by a general description of the basic materials and equipment used in a microbiological laboratory.

Each exercise is started with a summary of the theoretical knowledge, then the material and equipment need of the experiment are described, followed by a description of the experimental work. It is specified what questions should be answered in relation to the experiment performed and suggest how to evaluate the experiment.

Successful implementation of the experiments requires great accuracy and clarity. Generally, it is true for all type of laboratory work but it has special importance when we have to handle microorganisms safely.

Some experiments could be started at the same time and performed side by side so that they can be fully executed within the time available. Due to the nature of the microbiological work, the results can be evaluated in 24-48 hours after the experiments or even later, if it requires a prolonged incubation.

1.1. The regulations of the microbiological laboratory

The safety rules of general laboratory activities have to be respected in microbiological laboratory to avoid unwanted situations from working with electricity, gas, acids, poisonous and other dangerous chemicals. In addition, special precautions must be taken when using microbial cultures because of the risk of infection.

1.1. Any microbial culture used in the laboratory should always be handled as a pathogenic one!

1.2. It is strictly forbidden to eat or drink in the laboratory!

1.3. Do not use laboratory equipment for other purposes!

1.4. Laboratory tools and glassware have to be in perfect condition; do not use unsafe or broken items!

1.5. After completion of the experiments, the laboratory bench must be cleaned with a proper disinfectant.

1.6. Wash your hands before leaving the laboratory.

1.7. It is strictly forbidden to dispose microbial cultures in the drain. All such wastes have to be destroyed by heat or chemical treatment.

1.8. Do not pour melted agar medium into the drain!

1.9. Before washing, the used laboratory glassware should be disinfected by autoclaving or by chemical treatment.

1.10. You have to wear lab coat performing experiments in the laboratory. This coat should preferably not be used for other purposes.

1.2. Materials and laboratory tools

1.2.1. Media

Lots of different culture media are used for the cultivation of microorganisms in laboratory practice. Though the nutritional requirements are extremely variable between microorganisms, these media basically must contain the essential nutrients: carbon, nitrogen, mineral salts and water.

The classification of culture media can be based on various aspects.

1.2.1.1. According to the absence or presence of a solidifying agent

1.2.1.1.1. Liquid media (broth, nutrient solutions)

1.2.1.1.2. Solid media: complement the liquid medium with a solidifying agent.

Solidifying agents may include:

A) Agar-agar: a polysaccharide purified from seaweed; used in 1-3% concentration; hydrolyzed by very few microorganisms.

B) Gelatine: a polypeptide; used in 10-30% concentration; its melting point is a function of concentration; hydrolyzed by several microbes.

C) Silica gel: its advantage is that it does not contain organic components; preparation of the medium is time-consuming; rarely used.

1.2.1.2. According to the materials used

1.2.1.2.1. Undefined (Natural) media: based on organic materials whose exact composition is unknown, e.g., meat extract, yeast extract, malt, molasses, juices

1.2.1.2.2. Defined (Synthetic) media: their composition is well known, exactly defined.

1.2.1.2.3. Semi-synthetic media: mixtures of natural and synthetic components.

1.2.1.3. According to the nutrient components used

1.2.1.3.1. Basal media: except for the ingredients needed for growth, they do not contain any special additives.

1.2.1.3.2. Selective media: basal medium + selective inhibitor (e.g., antibiotic). Suppress the growth of certain microorganisms.

1.2.1.3.3. Differential or indicator media: basal medium + indicator compound that indicates specific metabolic reactions (e.g., lactose utilization, acid formation).

1.2.1.3.4. Special media: media supporting microbial growth for various special purposes.

Preparation of the culture medium: first weigh the solid constituents and then dissolve them in a total volume. After dissolution, set the final volume. The pH is adjusted with 5% HCl or with 5% NaOH with fine-grained indicator paper or instrument, then autoclaved. After autoclaving, the pH of the agar-containing media is reduced by a pH of 0.1 to 0.5 due to mild hydrolysis of the

solidifying agent. Agar-containing media can be sterilized under pressure, those, which contains gelatin flowing steam must be used (without pressure).
The heat-sensitive media components must be added to the autoclaved and properly cooled basal medium after sterilization by filtration.

1.2.2. Laboratory Glassware

All laboratory vessels used for microbial cultivation must be fitted with cotton swabs or other safe closure.

1.2.2.1. Test tubes: generally, 5 ml of liquid medium, 10 ml of solid medium for sloped agar, and 10 ml of solid medium for high agar, is required.

1.2.2.2. Flasks: for liquid microbial cultures most frequently Erlenmeyer flasks or flat bottom spherical flasks are used.

1.2.2.3. Special breeding vessels: Roux bottle and the Kolle flask; these provide large surfaces for the microbial culture.

The abovementioned glassware types are suitable for shaking and for standing liquid cultures. The culture vessels are sealed with a cotton/paper swab after loading the medium and then sterilized by autoclaving. It is advantageous to cover the cotton swab with e.g., aluminium foil before sterilization to prevent its moistening in the autoclave.

1.2.2.4. Petri dish: the most commonly used laboratory glassware. Sterilize the medium before pouring into a sterile petri dish. When pouring the medium, raise the lid only to the extent necessary. In a 9-10 cm diameter dish, 20-25 ml of medium is required.

1.2.2.5. The fermenter is a special laboratory equipment frequently with a large glass reaction vessel to cultivate microorganisms in large volume. The parameters (conditions) of cultivation can be ensured by appropriate control systems with data recording. Generally, it has a stirring system to ensure homogeneity of the microbial culture in the medium and auxiliary parts for controlled supplement of nutrients, gases, and other necessary additives.

In the laboratory, the culture vessels are marked as follows: 1.) the name of the microorganism, 2.) the date of inoculation, 3.) the type of medium, 4.) the number of the experiment, 5.) the name of the researcher.

Nowadays, practically all laboratory glassware has its disposable variant made of plastic. When performing experiments, special attention must be paid to the limit of their heat resistance.

2. Basic microbial techniques applied during the course

2.1. Inoculation
/- Practice 1 -/

Learning Objectives

Students will know

- what inoculation means
- simple tools for inoculation
- different inoculation techniques

I. Introduction

Under laboratory condition inoculation means the transfer of a pure culture into liquid or solid culture medium. Solid media immobilize cells and allow them to grow and form a visible mass of cells called colonies.

Loops are simple tools widely used for inuculation. It can be used for inoculation of liquid or solid media as well in different types of cultivation dishes. Working with deep-agar, needles are used to inoculate the media.

During the proccess of inoculation a series of steps will prevent contamination during the transfer of the cells: flaming the loops or needles and the surface of the cultivation tube sterilize them.

During inoculation, work always close to the flame because the warm rising air prevent contamination.

Required materials

Microorganisms: *Saccharomyces cerevisiae* culture on slant agar

Materials: sterile slant agar, loop

Method

Follow the next steps for taking out the inoculum:

1. Flame the loop (Figure 1.A)
2. Remove the cap of the tube contaning the culture (Figure 1.B-C)
3. Flame the tube tip (Figure 1.D)
4. Take out the inoculum (Figure 1.E-F)
5. Flame the tube tip again (Figure 1.G-H)
6. Recap the tube (Figure 1.I)

Remove the the cap of the sterile tube containing the slant agar. Flame the tube tip. Transfer the inoculum onto the surface of the slant agar. Flame the tip of the tube and recap it. Flame the loop (Figure 1.J).

Write the name of the strain onto the tube. Incubate the culture for 48 hours at 30 °C.

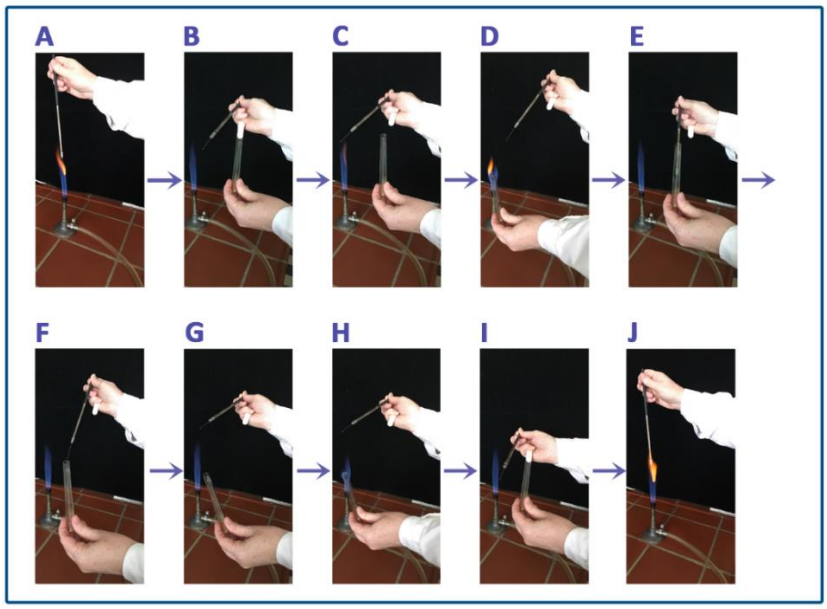

Figure 1. Taking out the inoculum of the culture

Check your progress

- **Prepare agar slants and inoculate coloured bacterial strains to them! Check the quality of the cultures after 2 days of incubation!**
- **What does inoculation refer to?**
- **What kind of tool is suitable for inoculation of deep-agar?**

2.2. Dilution

/- Practice 2 -/

Learning Objectives

Students will know

- how to prepare a dilution series of a suspension
- how the cell number of the population will change during the dilution

I. Introduction

When the cell number of a liquid culture or a suspension is too high it is necessary to dilute it. This practice provides an example for preparing tenfold dilution seies.

Required materials

Microorganisms: *Saccharomyces cerevisiae* 24-hour-old liquid culture

Materials: pipette, pipette tips, sterile glass tubes, sterile distilled water

Method

Prepare a serial dilution as shown in Figure 1. Fill 6 glass tubes with 4.5 ml sterile distilled water. Add 500 µl liquid culture to the first tube. Vortex it, take out 500 µl suspension and add it to the next tube. Follow this steps until the 6^{th} tube. The cell number of the original culture decreases with point tenth at each step.

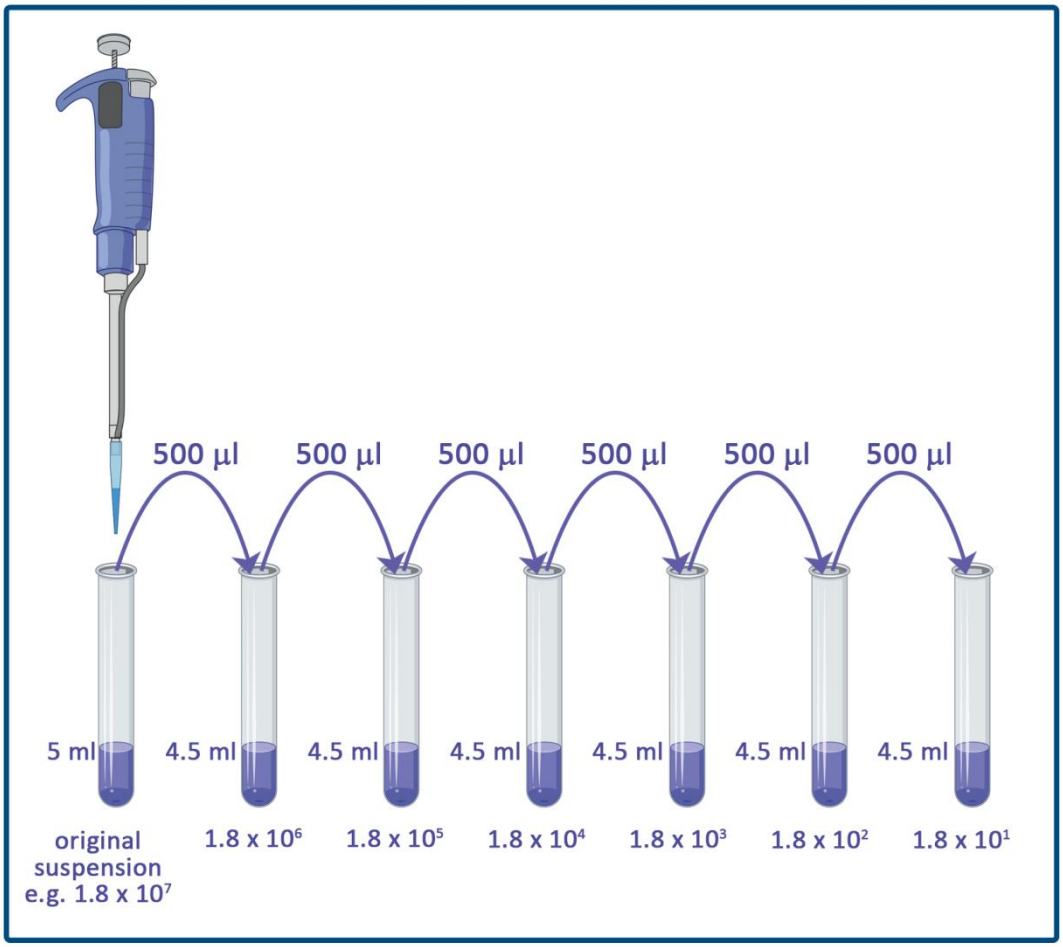

Figure 1. Preparation of dilutions

Check your progress

- **A 10-fold dilution series in 6 steps was prepared from a *Saccharomyces cerevisiae* suspension. The total cell number of the original suspension was estimated as 6.3×10^7 cells/ml. Establish the cell number in the 5th step of the dilution series!**

2.3. Total cell count of a yeast population
/- Practice 3 -/

Learning Objectives

Students will know

- how to use the Bürker chamber
- a simple method to determine the total cell number of a yeast population by a direct cell counting method

I. Introduction

There are direct and indirect methods to determine the cell number of a microbial population. Direct methods count directly the cells in the population therefore refer to the total cell number (dead and living cells together) indirect methods assess a property of the population what is proportional to the cell number.

This practice demonstrates a direct cell counting method to determine the total cell number of a yeast population.

Counting chambers are generally used to establish the number of the suspended particles (bacterial or yeast cells, fungal spores) in a given volume. Counting chambers are microscopic slides with grooved cross-channels. The middle part of the slide is thinner with standard size, so the height of the liquid column between the slide and the cover slip is known. As the size of the grid is also known, the volume between the slide and cover slip can also be defined.

The height of the more widely used Bürker-chambers (Fig. 1.) is 0.1 mm. The cross-channels divide the slide into big and small squares and rectangles (Fig. 1.b).

The **area** of the

big square is 1/5 mm x 1/5 mm = 1/25 mm^2,

rectangle is 1/5 mm x 1/20 mm = 1/100 mm^2

small square is 1/20 x 1/20 mm^2 = 1/400 mm^2.

As the height of the chamber is 0.1 mm the **volume** of

the big square is 1/250 mm^3,

the small square is 1/4000 mm^3,

the rectangle is 1/1000 mm^3.

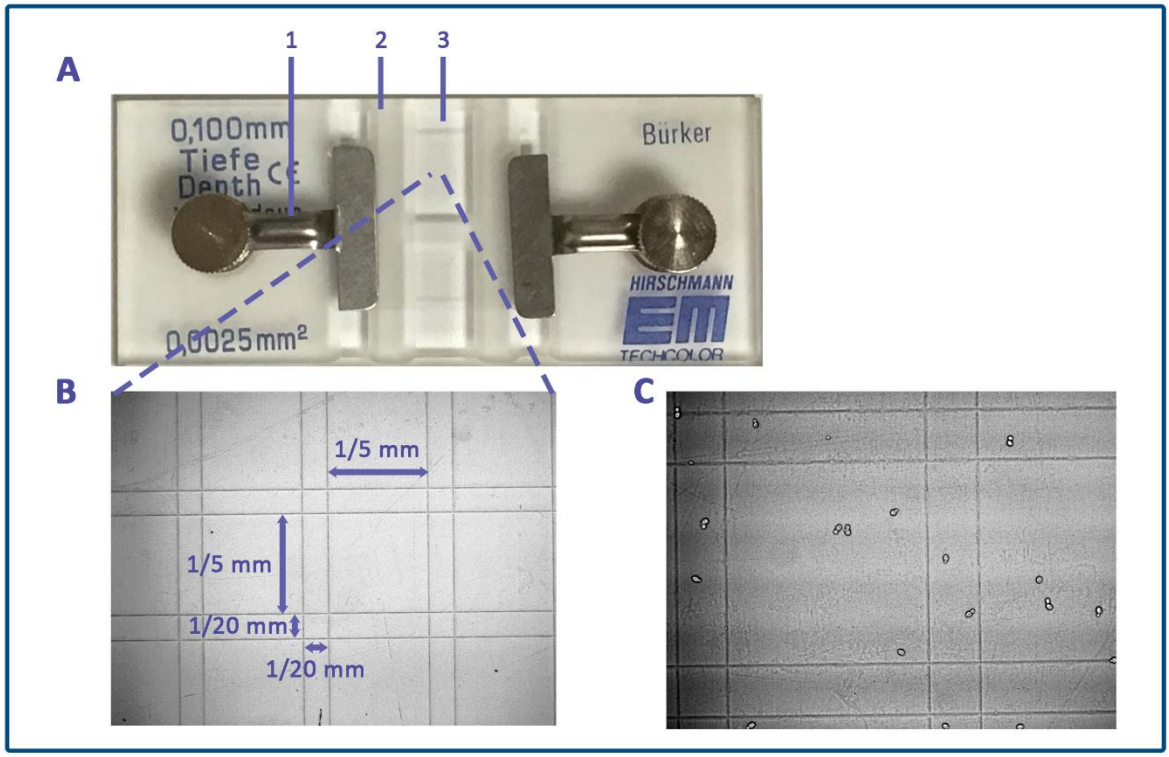

Figure 1. Bürker-chamber
(A) Parts of the chamber 1. clamp 2. cover glass 3. counting chamber (B) Enlarged grid (C) Enlarged grid with yeast cells.

Required materials

Microorganisms: *Saccharomyces cerevisiae* 24-hour-old liquid culture

Materials: pipette, pipette tips, sterile glass tubes, sterile distilled water, Bürker chamber, cover slip, light microscope

Method

Clean the Bürker chamber with a detergent and rinse it in distilled water. After drying, place the cover slip onto the surface of the chamber and secure it tightly until Newton's rings appear. Put a drop of the suspension near to the cover slip. The suspension will be absorbed under the cover

slip due to the capillary action. Focus the microscope on the area of the chamber and count the cells in one unit (either small or big squares, rectangles)! Repeat the process for 10 units! Calculate the average!

The total cell count should be given for 1 ml (= 1 cm^3)!

Potential pitfalls:

Use always the same type of unit for the counting!

If the suspension contains countless cells dilute the yeast suspension! In this case you have to take the rate of the dilution under consideration calculating the cell number of the original suspension!

Example 1:

You obtained the following counts in big squares: 8, 3, 4, 2, 7, 7, 5, 11, 3, 9

Calculate the average (x):

$$x = \frac{8+3+4+2+7+7+5+11+3+9}{10} = 5.9$$

The volume of the big squares is 1/250 mm^3.

Estimate the mean cell number of the suspension using the following formula:

$$X = \frac{5.9 \times 1000}{\frac{1}{250}} = 5.9 \times 1000 \times 250 = 1475000$$

In an abbreviated form: $X = 1.475 \times 10^6$ cells/ml

Example 2:

The original suspension contained countless number of cells therefore you have made 50 fold dilution, and counted the cell number in the diluted sample.

You obtained the following counts in rectangles: 6, 2, 7, 4, 5, 7, 12, 9, 3, 9

The average (x) is calculated:

$$x = \frac{6+2+7+4+5+7+12+9+3+9}{10} = 6.4$$

The volume of the rectangles is 1/1000 mm^3.

Estimate the mean cell number of the original suspension using the following formula:

$$X = 50 \times \frac{6.4 \times 1000}{\frac{1}{1000}} = 50 \times 6.4 \times 1000 \times 1000 = 320000000$$

In an abbreviated form: $X = 3.2 \times 10^8$ cells/ml

Check your progress

- **Compare the direct an the indirect cell counting methods!**
- **What do direct cell counting methods refer to?**
- **What can counting chambers is used for?**
- **Estimate the total cell number of a yeast population! The cell counts in rectangles were as follows: 12, 4, 6, 3, 5, 13, 9, 5, 8, 14.**
- **Estimate the total cell number of the yeast population! The cell counts in small squares were as follows: 2, 2, 3, 0, 5, 4, 0, 3, 2, 1.**
  As the original suspension was too dense, these counts were made in 100-fold dilution.

2.4. Staining method for cell and ascospore differentiation in a *Saccharomyces cerevisiae* population /- Practice 4 -/

Learning Objectives

Students will know

- what differential staining means
- what aggressive staining method means and what it is good for
- a simple method for staining *S. cerevisiae* cells and ascospores

I. Introduction

To observe microbial cells one needs a microscope. Bacteria, fungi, yeasts, unicellular algae and parasites can be examined under optical microscope. This type of microscope works with lower magnification therefore it is suitable for visualization of larger objects only (viruses cannot be observed under this type of microscope!). The cells are visible under optical microscope because they absorb and scatter the light differently from the surroundings. However, the contrast between the cells and the background can be elevated by using specific dyes what stains the cells or cell compartments but not the surroundings.

To stain microbes, generally positive staining methods are used. During these methods the objects are stained. The positive staining methods can be separated into simple and differential staining methods. During simple staining methods the cells are stained with one dye only. The commonly used dyes (e.g. safranin) have positive charge therefore they are called basic dyes. Basic dyes strongly bind to negatively charged macromolecules (e.g. nucleic acids, proteins).

During differential staining methods two dyes with different color are used to distinguish between the cell types. One dye is used as a primary stain and the other one is applied for counterstaining.

Spores (endospores of bacteria or different spore types of fungi) are usually resistant to simple staining techniques. Therefore, so called “aggressive” method is used to stain them. During this technique the dye (often phenol-containing) is forced to penetrate into the spores by heating.

Experienced users can observe vegetative cells and ascospores in a *S. cerevisiae* population under bright field microscope. For students, to use differential staining is more ubiquitous. During this practice the Schaeffer-Fulton method will be used to differentiate between the vegetative cells and ascospores of *S. cerevisiae*. Malachite green will be used as primary dye and safranin as a counterstain. The sample will be visualized under bright field microscope.

Required materials

Microorganisms: *Saccharomyces cerevisiae* seven-day-old culture on sporulation medium

Materials: malachite green solution, safranin solution, slides, cover plates, ethanol-ether (1:1) mix, distilled water, filter paper, staining tub, water bath, light microscope

Malachite green solution:

1 g malachite green

1 g phenol

100 ml distilled water

Safranin solution:

6 ml 5% safranin O

10 ml 3% KOH

5 ml 87% glycerol

79 ml distilled water

Method

Clean the slide with a detergent and rinse it in distilled water. After drying, place a drop of distilled water onto the slide and prepare the smear: Spread the culture in a thin film. Let it air dry then fix the cells onto the surface of the slide with flooding the smear with ethanol-ether mix. After the evaporation of the fixation mix take a piece of filter paper and cover the smear with it.

Saturate the blotting paper with malachite green and place the slide over a boiling water bath for 10-15 minutes. Keep the paper moist by adding more dye as required. Remove the filter paper and decolorize the sample with water then counter-stain it with safranin: flood the smear with safranin solution for 30 seconds. Wash it with water and after drying it can be visualized under the microscope. Ascospores have to be green, vegetative cells have to be red.

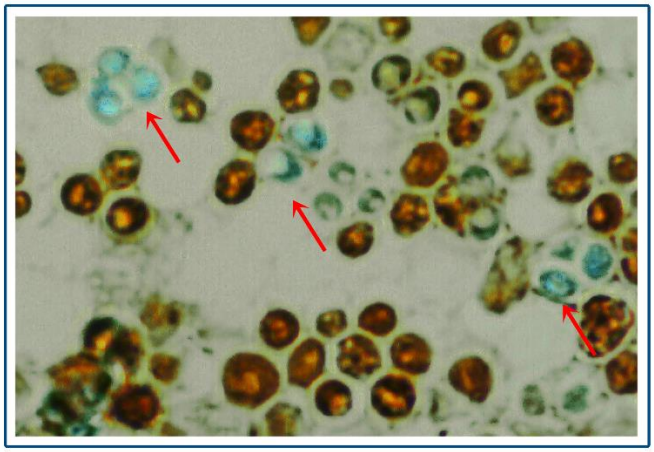

Figure 1. *Saccharomyces cerevisiae* cells and ascopores.

Safranin-stained vegetative cells (red) and malachite green-stained ascospores (green).

Check your progress

- Briefly characterize each positive staining method!
- Why can basic dyes be applied to stain microbial cells?
- What kind of technique is suitable for staining the spore?
- Outline the Schaeffer-Fulton staining method, how it is used?

3. Advanced Microbiological Practices

3.1. Studies On Microbiological Genetics

3.1.1. Sexual cycle of *Saccharomyces cerevisiae* / Practice 5-10 /

Learning Objectives

Students will know

- what is homothallism and heterothallism
- what does haplo-diploid life cycle refer to
- what kind of methods are available to study the segregation of the genetic markers in the progenies

I. Introduction

The haploid *Saccharomyces cerevisiae* has two different mating types 'a' and 'α'. Separately the cells of the different mating types can be divided by budding (Figure 1). Budding is a mitotic division, during this process the number of the chromosomes does not change.

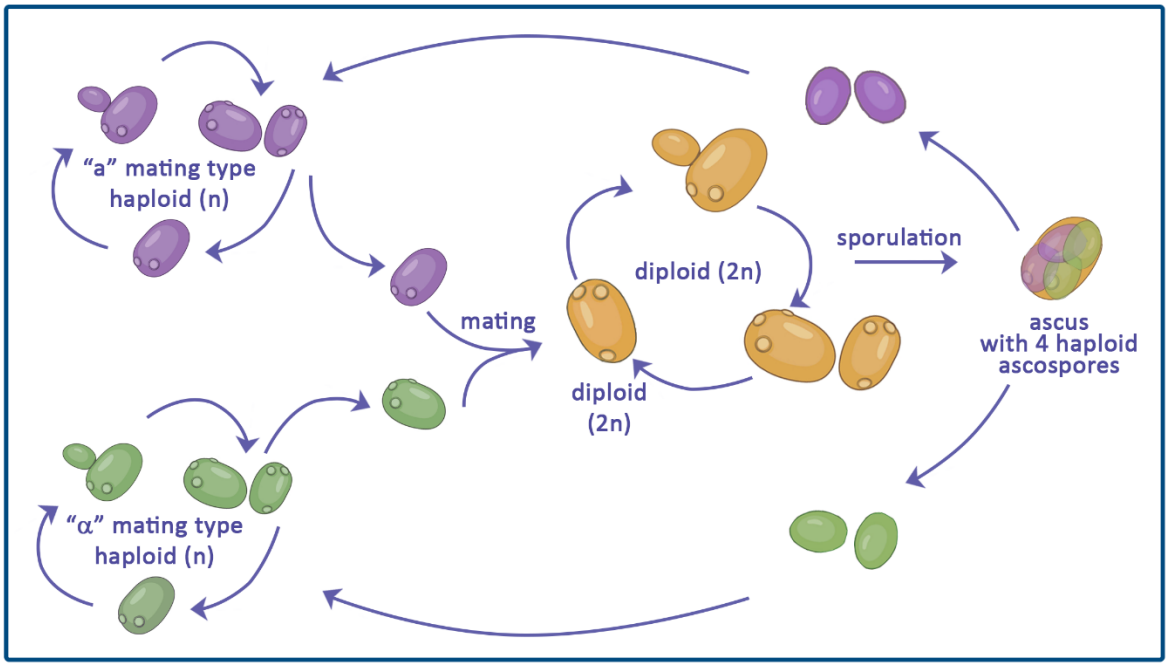

Figure 1. Life cycle of *S. cerevisiae*

The ‘a’ cells produce and secrete ‘a’ factor, ‘α‘ cells produce ‘α‘ factor. Both ‘a’ and ‘α‘ factors are pheromones and both are able to bind to the cell surface receptors of the opposite mating type. If the cells of the two opposite mating types are mixed, the pheromones induce morphological changes in the cells finally they will fuse (this process is called conjugation) creating diploid zygotes (Figure 2). Diploid cells can divide by mitotic division (budding) establishing a diploid cell population. As vegetative cells can be haploid or diploid and both haploid and diploid cells are able to undergo mitotic division the life cycle of *Saccharomyces cerevisiae* is called haploid-diploid life cycle.

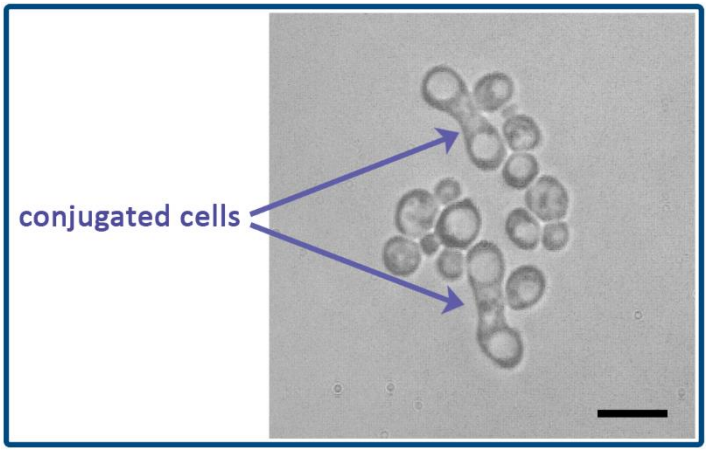

Figure 2. Conjugated cells

Diploid cells can become a zygote and the zygote can undergo meiotic division under starvation. The result of the meiotic division is an ascus with 4 haploid ascospores in it (tetrad). The distribution of the mating type alleles in the ascus is: 2 a: 2 α. When the wall of the ascus disrupts the ascospores deliberate and will be haploid vegetative cells those start to propagate by budding.

The genetic analysis of ascospores in one ascus is called tetrad analysis. One needs micromanipulator to carry out this study. The other way of the genetic analysis is called random spore analysis. In this case a population of ascopores is under the study independently of their position in the ascus.

Haploid *S. cerevisiae* cells are able to change their mating type the process is called mating type switching. The mating type of the cell is determined by the allele located in the MAT locus. MAT locus is situated on the 3rd chromosome of *S. cerevisiae*. Two silent gene locus MATa and MATα can be found on this chromosome as well. The mating type switching occurs when the allele in the active MAT locus is replaced by one of the two silent gene. The strains what are able to change their mating type are called homothallic, because pure culture derived from a single cell is able to sporulate as a consequence of the switching. Those strain what are not able to

switch their mating type are called heterothallic and the pure culture derived from single heterothallic cell will never sporulate.

Heterothallic strains are useful during genetic crossing experiments because conjugation of the strains can be controlled more easily comparing to homothallic strains.

3.1.1.1. Conjugation
/- Practice 5 -/

Required materials

Microorganisms: Fourteen-hour-old shaken culture of *Saccharomyces cerevisiae* strains 0666 (a, *ura3*, *ade1*) and GRF-18 (α, *leu2*, *his3*) in 20 ml YPD. Twenty-four-hour-old mixed (1:1) culture of the two strains.

Media:

YPD: 0.5% yeast extract, 1% pepton, 1% D-glucose, 2% agar

Minimal medium: 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 1% glucose, 0.1% Wickerham vitamin solution, 2% agar

Materials: pipette, pipette tips, sterile glass tubes, sterile distilled water, Bürker chamber, cover slip, light microscope, microcentrifuge, Petri dishes, ethanol,

Method

A. Count the cells of the fourteen-hour-old shaken culture of strains 0666 and GRF-18 in Burker chamber. Pipette 10^7 cells from each culture in a sterile Eppendorf tube. Pellet the cells by centrifugation (5 min, 5000 rpm) after that re-suspend the cells in 1 ml YPD and incubate them for 2 hours at 30 ˚C. Follow the conjugation process under the microscope!

B. Take out $2x10^7$ cells from the twenty-four-hour-old mixed (1:1) culture in a sterile Eppendorf tube. Pellet the cells by centrifugation (5 min, 5000 rpm) after that re-suspend the cells in 1 ml sterile water and pellet the cells again (5 min, 5000 rpm).

Re-suspend the cells again in 1 ml sterile water and prepare a tenfold dilution series in four steps. The dilution can be made in sterile water.

Spread 50 µl aliquots from the original suspension and the 10x and 100x dilutions to the surface of minimal medium (2 paralels) to select diploid cells.

Spread 50 µl aliquots from the 1000x and 10000x dilutions to the surface of YPD medium (2 paralels) as control.

Incubate the plates for 48 hours at 30 ˚C. Establish the frequency of the conjugation after the incubation.

3.1.1.2. Diploid isolation
/- Practice 6 -/

Required materials

Microorganisms: colonies grown on minimal medium

Materials: 4 slant agar made of YPD medium, loop

Method

Isolate 4 colonies from the minimal medium (these are diploid cells!) inoculate them onto slant agar and incubate them for 6 days at 30 ˚C.

3.1.1.3. Induction of the sporulation /- Practice 7 -/

Required materials

Microorganisms: cultures on slant agar inoculated on the previous practice

Media

PSM (presporulation medium): 0.8% yeast extract, 0.3% pepton, 10% D-glucose

SM (sporulation medium): 0.5% Na-acetate, 0.5% KCl, 0.1% yeast extract, 0.05% D-glucose, 2% agar

Materials: sterile centrifuge tube, microcentrifuge, pipette, tips

Method

One day before the practice inoculate the selected diploid strains into PSM medium and incubate them for 24 hours at 30 ˚C.

Pellet the cells by centrifugation (5 min, 5000 rpm) after that re-suspend the cells in 1 ml sterile water and pellet the cells again (5 min, 5000 rpm).

Re-suspend the cells in 1 ml sterile water and inoculate 25 µl onto the surface of SM medium. Dry the surface under sterile box. Incubate the plates for 1 week at 27 ˚C.

3.1.1.4. Ascospore isolation /- Practice 8 -/

Required materials

Microorganism: cultures on SM medium inoculated in the previous practice

Medium: 2xYPD: 1% yeast extract, 2% pepton, 2% D-glucose, 2% agar

Materials: sterile distilled water, loop, microcentrifuge tubes, water bath, snail gut enzyme solution (5% in sterile distilled water), Triton X-100 (0.1%), sterile glass beads (0.5 mm diameter), ethanol, slides, cover slips, microscope

Method

Check the ascus formation under the microscope. Select the strain with several ascus and make a dense suspension in 0.5 ml sterile water in a sterile test tube. Place the suspension to a water-bath at 55 °C for 20 min. During this step the vegetative cells will die.

Add 1.5 ml snail gut enzyme to the suspension and incubate it at 30 ˚C until the lysis of the ascus wall. Check the process under the microscope!

Add 1/5 volume of sterile glass beads to the suspension and vortex it to separate the ascospores from each other.

Pellet the beads and pipette 1 ml of the supernatant into a sterile microcentrifuge tube. Centrifuge it (5 min, 5000 rpm) and wash the cells with 0.1% Triton X-100 solution, centrifuge again (5 min, 5000 rpm).

Suspend the cells in 1 ml sterile water and prepare a tenfold dilution series in three steps (10x, 100x, 1000x). Spread 50 µl from each dilution onto the surface of 2xYPD medium in two replicates. Incubate the plates for 72 hours at 30 ˚C.

3.1.1.5. Random spore analysis /- Practice 9 -/

Required materials

Media

A. Minimal medium: 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4 , 0.05% $MgSO_4$, 1% glükóz, 0.1% Wickerham vitamin solution, 2% agar supplemented with uracil, leucine, histidine and adenine.

B. Minimal medium supplemented with uracil, leucine and histidine

C. Minimal medium supplemented with uracil, leucine and adenine

D. Minimal medium supplemented with adenine, leucine and histidine

E. Minimal medium supplemented with uracil, adenine and histidine

The concentration of leucine, histidine and uracil: 30 µg/ml, adenine: 5 µg/ml.

Materials: sterile velvets

Method

Choose the plates with countable number of colonies and transfer the colonies to the surface of the supplemented minimal medium with sterile velvets (replica plating).

Incubate the plates for 72 hours at 30 ˚C.

3.1.1.6. Genetic analysis
/- Practice 10 -/

Required materials

Replica plates from Practice 5.

Method

Establish the distribution of the genetic markers among the progenies (pr)!

Cross: GRF-18 X 0666							
Media / Strains	MM uracil Ade Leu His	MM Ade Leu His	MM uracil Leu His	MM uracil Ade His	MM uracil Ade Leu	PHENO -TYPE	GENOTYPE
GRF-18							
0666							
pr 1							
pr 2							
pr 3							
pr 4							
pr 5							
pr 6							
pr 7							
pr 8							
pr 9							
pr 10							
pr 11							
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pr 42							
pr 43							
pr 44							
pr 45							
pr 46							
pr 47							
pr 48							
pr 49							
pr 50							

Check your progress

- What does homothallism/heterothallism mean?
- Why do we use heterothallic strains during the practice?
- How the diploids can be selected?
- What is the principle of the diploid selection?
- How can the sporulation be induced?
- What method is suitable to transfer several colonies in the same time to a plate?
- What are the methods suitable for genetic analysis of the progenies?
- What is the phenotype of the *ade1* or *ade2* mutants in *S. cerevisiae*?
- Could you establish linkage between the analysed genetic markers?

3.1.2. Study of the killer system of *Saccharomyces cerevisiae* /- Practice -11-12 /

Learning Objectives

Students will know

- what are the killer toxins
- what is the mode action of the killer toxins
- what is the genetic background of the toxin production

I. Introduction

The killer yeast system was first described in 1963. Killer yeast such as *Saccharomyces cerevisiae* is able to secrete a toxic protein, which is lethal to receptive cells. The killer yeast cells are immune to the toxic effects of the protein due to an intrinsic immunity.

In *S. cerevisiae* the toxins encoded by a double-stranded RNA virus, translated to a precursor preproprotein. After maturation, the preprotoxin is secreted outside of the cells, where it may affect susceptible cells.

The virus, L-A, is an icosahedral virus of *S. cerevisiae* comprising a 4.6 kb genomic dsRNA segment. L-A virus has several satellite double-stranded RNA viruses, called M particles (M1, M2, M28). The genomic segment of L-A encodes for the viral coat protein and a protein which replicates the viral genomes (dsRNA-dependent RNA polymerase). The M dsRNAs encode the toxin, what has at least three variants in *S. cerevisiae* (K1, K2, K28).

The initial protein product resulted by translation of the M dsRNA is the so-called preprotoxin, which is targeted to the yeast secretory pathway. The preprotoxin is processed and cleaved to produce an α/β dimer, which is the active form of the toxin, and is released into the environment. The two most studied variant toxins in *S. cerevisiae* are K1 and K28.

K1 binds to the β-1,6-D-glucan receptors on the target cell wall, moves inside, and then binds to the plasma membrane receptors. It forms a cation-selective ion channel in the membrane, which is lethal to the cell. The K1 toxin acts in narrow pH range (pH: 3-5). The dead

leaky cells can be stained by methylene blue (Figure 1.) because this dye with small molecular weight can enter to the dead cells across the pores opened by the toxin.

K28 uses the cell wall α-1,6-mannoprotein receptors to enter to the cell. From the ER, K28 moves into the cytoplasm and shuts down DNA synthesis in the nucleus, triggering apoptosis.

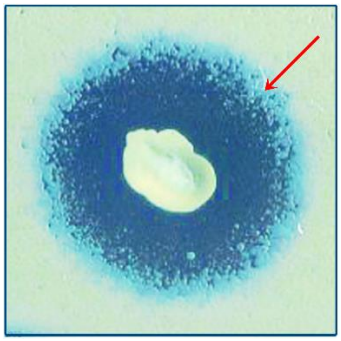

Figure 1. Killer activity of *S. cerevisiae*

Sensitive cells were inoculated into the background. The toxin diffused into the medium and caused a wide inhibition zone around the killer strain. The methylene blue supplemented in the medium stained the dead sensitive cells (red arrow).

3.1.2.1. Killer activity /- Practice 11 -/

Required materials

Microorganisms: Four-day-old shaken culture of *Saccharomyces cerevisiae* strain T158C (K1 toxin producing strain) in YPD medium and one-day-old *S. cerevisae* S6 (K1 toxin sensitive) strain on slant agar.

Media:

T-28A: 0.5% yeast extract, 1% pepton, 1% D-glucose, 2% agar

Citrate/phosphate buffer (pH 4.2)

methylene blue solution (0.3 mg/ml)

Materials: Microtubes, pipettes, tips, proteinase K solution (10 mg/ml), water bath, sterile tube, sterile water, Petri dish, sterile borer, loop, microcentrifuge

Method

Melt the T-28A media in a pressure cooker. Adjust the pH of the medium to 4.2 with citrate buffer (T-28B). Add some drops of methylene blue (T-28C) to the medium and pour one plate.

Prepare a dense suspension of *S. cerevisiae* S6 (killer sensitive strain) in 3 ml sterile distilled water. Make a massive inoculation on the plate. Make 4 wells with a sterile borer in the inoculated plate.

Centrifuge 1 ml suspension of *S. cerevisiae* T158C (K1 toxin producing strain) in a sterile Eppendorf tube (5 min, 5000 rpm). Pipette 200 µl supernatants into 3 sterile Eppendorf tubes. One will be the control it will be loaded to one well. Add 2 µl Proteinase K to the second Eppendorf tube and incubate it at 37 ˚C for 30 min. After the incubation load the sample to the second well. Place the third Eppendorf tube to a hot water bath for 30 min. Load the sample to the third well after 30 min. Pipette cells of the killer strain to the 4^{th} well.

Incubate the plate at room temperature for 24 hours.

3.1.2.2. RNase protection assay /- Practice 12 -/

Required materials

Microorganisms: Four-day-old shaken culture of *Saccharomyces cerevisiae* strain T158C (K1 toxin producing strain) in YPD medium

Materials: Microtubes, pipettes, tips, RNase solution (10 mg/ml), water bath, centrifuge tubes, sterile phosphate buffer (0.1 M), phenol:chloroform:iso-amyl-alcohol 25:24:1, loading buffer, agarose, TBE buffer, ethidium-bromid, sterile distilled water, cylinder, scale, gel electrophoresis unit, microcentrifuge

Method

Take 50 ml three-old-day culture of *S. cerevisiae* T158C. Pellet the cells by centrifugation (10 min, 3000 rpm, 4°C). Suspend the cells in 5 ml ice-cold phosphate buffer (0.1 M, pH: 7.4) and disrupt them in French pressure at 20000 Ψ. Pellet the cells and cell debris by centrifugation (30 min, 15000 rpm, 4°C).

Pipette 200 µl supernatant in 3 Eppendorf tubes.

Tube 1.: Add equal amount of PCI (phenol:chloroform:iso-amyl-alcohol 25:24:1), centrifuge it (10 min, 10000 rpm, room temperature). Pipette 50 µl from the upper phase to a new Eppendorf tube. Add 10 µl bromo-phenol blue to the sample and load it to a well of an agarose gel.

Tube 2.: Add equal amount of PCI (phenol:chloroform:iso-amyl-alcohol 25:24:1), centrifuge it (10 min, 10000 rpm, room temperature). Pipette 50 µl from the upper phase to a new Eppendorf tube. Add 1 µl RNase to the sample and incubate it at 37°C for 30 min. After the incubation time add 10 µl bromo-phenol blue to the sample and load it to a well of an agarose gel.

Tube 3.: Add 2 µl RNase to the sample and incubate it at 37°C for 30 min. After the incubation time add equal amount of PCI (phenol:chloroform:iso-amyl-alcohol 25:24:1), centrifuge it (10 min, 10000 rpm, room temperature). Pipette 50 µl from the upper phase to a new Eppendorf tube. Add 10 µl bromo-phenol blue to the sample and load it to a well of an agarose gel.

Gel electrophoresis

Prepare an agarose gel: 0.7% agarose, 100 ml TBE buffer. Melt the agarose in microwave oven and add 10 µl etidium bromide to the liquid solution. Pour the gel.

After solidification load the samples to the wells and run the gel at 70 V for 45 min.

Check your progress

- What is the mode of action of K1 toxin?
- What are the optimal conditions for toxin activity?
- What is the role of the methylene blue in the experiment?
- How can the chemical nature of the toxin be determined?
- What is the genetic background of killer toxin production in *S. cerevisiae*?
- What „RNase protection assay" is good for?
- What method is suitable to open the yeast cells?
- What proteins are coded on L-A virus?

3.1.3. *General introduction to the asexual, sexual and parasexual life cycles of the filamentous fungus model organism, Aspergillus nidulans*

Introduction of the model organism Aspergillus nidulans

The filamentous fungus *A. nidulans* has many advantageous characteristics, which make this fungus an excellent candidate for studying life cycle, genetic interactions, various molecular biological processes (sensing and signal transduction of environmental factors and response to them; regulation of conidiogenesis and -ascosporogenesis; regulation and biosynthesis of primary- and secondary metabolites; DNA metabolism; organisation of actin and cytoskeleton; regulation and molecular events of polarized growth; transport mechanisms; mitochondrial functions; role of remodeling complexes, activators and repressors in chromatin functions and many more...), physiological functioning and evolution. These characteristics are the following.

- It has minimal nutritional requirements and in that one source of organic carbon (glucose, lactose, galactose, saccharose, mannose, ethanol, certain amino acids and many more...), one source of nitrogen (nitrate, ammonium, acetamide, hypoxanthine, xanthine, uric acid, allantoin, urea and many more...), inorganic salts and a few trace elements are needed. This simplicity supports the studies on the genetics of biosyntheses.
- It grows rapidly, with which the fungus produces great amount of biomass in a few days that could be subjected to experimental processing.
- It has filamentous growth.
- It can be cultured in liquid medium.
- It performs a compact growth with easily accessible asexual and sexual reproductive structures. The sexual structures (cleistothecia) are closed and remain closed even when the ascospores are ripe. Therefore cleistothecia with viable ascospores can be preserved for years.
- Metabolic versatility with wide range of primary- and secondary metabolic pathways.
- It performs complete sexual-, asexual and parasexual life cycles.
- It is self-fertile due to homothallism.
- Its uninucleate conidiospores and the clonal reproduction make possible the rapid purification of mutants or transformants obtained by genetic crosses, mutagenesis or transformation; detection of cytoplasmic inheritance; isolation of artificially induced diploids; measurement of mutation rates; selection of somatic recombinants.

- Its haploid genome makes mutants easily selectable and execution of Mendelian analysis within one generation.
- Heterokaryons and diploids can be easily isolated for complementation tests, dominance tests and study of somatic crossing overs.
- The availability of dozens of auxotroph mutants for genetic and molecular biological studies.
- Its transformation and genetic manipulation is easy that support the application of molecular biological methods.
- Its whole genome is sequenced and annotated (http://www.aspergillusgenome.org).

Life cycles of A. nidulans

The *A. nidulans* is a homothallic filamentous fungus. Its genome carries both the MAT1-1 and MAT1-2 mating type genes, with orthologues corresponding to the two oposite mating type genes in the heterothallic closely-related species, such as *Podospora anserina*. Since both mating type genes are present and expressed in the genome, *A. nidulans* is able to accomplish the full sexual cycle itself, without the need of a mating partner. When this happens, we call the process selfing, inbreeding, or more accurately, homozygous cross (Figure 1). As a result of the selfing the nuclei of the reproductive structures, the ascospores, are identical with that were found in the parental hyphae. The heterozygous sexual cycle (outcross) starts with a heterokaryon formation by anastomosis between the hyphae of two, genetically different mycelia. The result of the heterozygous sexual cycle is the production of both recombinant and parental types of ascospores (Figure 2).

A. nidulans colonies can be formed by the germination of either the asexually produced conidiospores or the sexually produced ascospores (Figure 1). Ascospores are binucleate (each contains two identical nuclei) but the conidiospores are uninucleate. The colony, which develops from the germination of a single ascospore or a conidiospore, is homokaryotic. This means that all the nuclei in the hyphae are identical.

<u>Asexual reproduction in homokaryotic mycelium</u>

When a homokaryotic mycelium grows for 40-48 hours in nutritionally rich environment, the asexual reproduction (conidiogenesis) will be initiated when the colony receive a light stimulus. The conidiogenesis starts with the differentiation of certain hyphal compartments to foot cells. The foot cells start to form an upward growing stalk from the middle of the compartment (Figure 1). The purpose of this upward growth is to push the reproductive structures high above the mycelium mat in order to their efficient spreading by the air. At the tip of the stalk a vesicle is

formed, which is full with nuclei (Figure 1). Metula cells forming a cell layer on the surface of vesicle are generated by yeast like mitotic division-, budding of the nuclei in the vesicle (Figure 1).

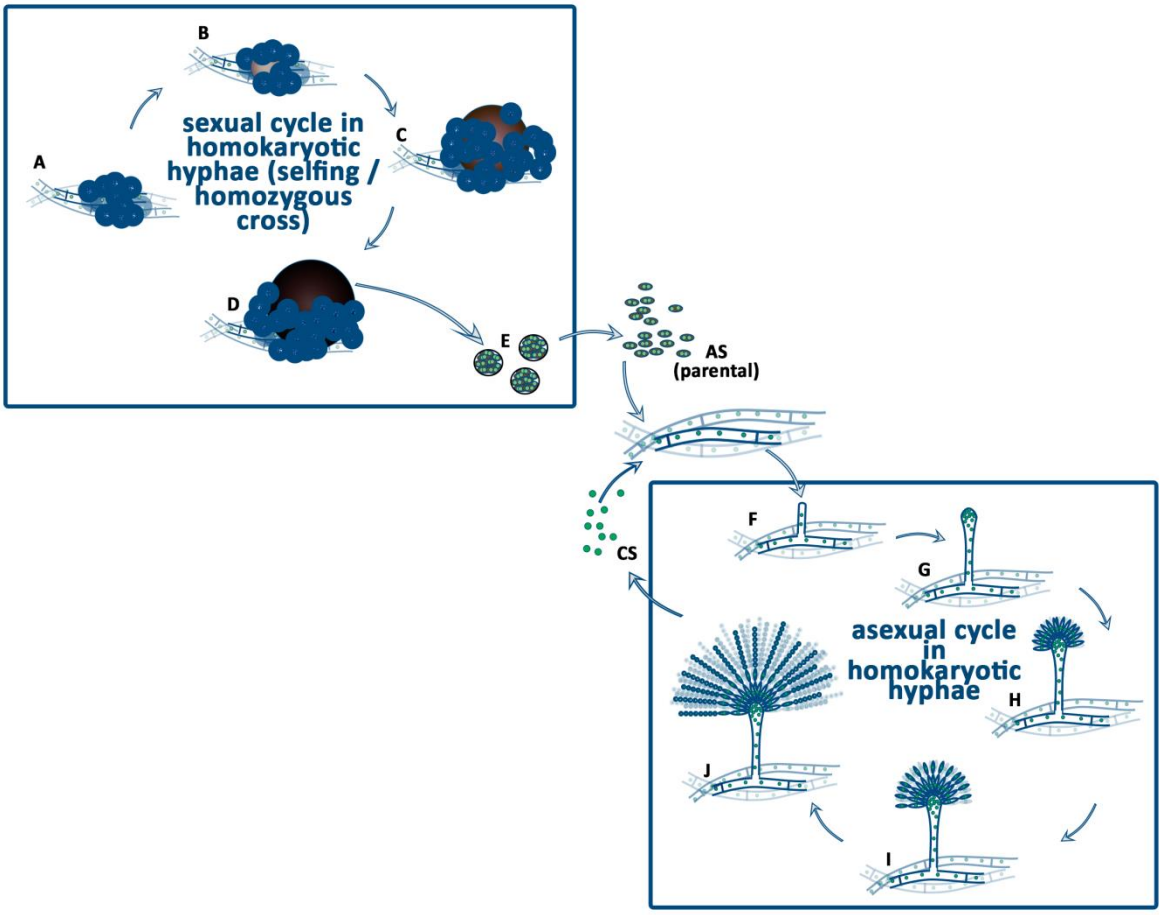

Figure 1. Life cycle of *A. nidulans* homokaryotic hyphae.
The homozygous sexual cycle of *A. nidulans* (A-E) begins with the production of Hülle cells when the environmental conditions are supportive (A). Surrounded by the Hülle cells, ascogenous hyphae are differentiated (B). These ascogenous hyphae form a ball shape nest, which is called primordium (B). Around the ball of ascogenous hyphae flattened longitudional hyphae are differentiated to the pericarp of the cleistothecium. The primordium grows and forms the µ-cleistothecium, in which the ascogenous hyphae start to develop ascus mother cells and asci, where meiotic events occur (C). Upon maturation, the µ-cleistothecium hardens its pericarp that is accompanied by a dark pigment accumulation of the pericarp cells, while hundred thousands of asci are formed inside the cleistothecium (D). Each ascus contains eight binucleate, parental type ascospores with dark red color. The asci and the free ascospores (AS) are released to the environment. Germination of an ascospore will result in a homokaryotic mycelium formation. The homozygous asexual cycle (the conidiogenesis) starts with the differentiation of a hyphal compartment into a foot cell, which starts to grow upward (forms the stalk), high above the mycelial mat (F). At the tip of the stalk a vesicle is formed (G). Through budding of the nuclei in the vesicle metula cells are formed (H). The metula cells are uninucleate and by covering the surface of vesicle, they form the layer of the primary sterigmata (H). Through the budding of the metula cells the secondary sterigmata layer composed of uninucleate phialide cells is formed (I). Each of the phialide cells starts to produce uninucleate conidiospores, by subsequent repetition of budding. As a result, chains of conidiospores (CS) are formed by the phialide cells (J). Germination of a conidiospore will result in a homokaryotic mycelium formation.

The metula cells are uninucleate. The layer of metula cells covering the vesicle is called primary sterigmata. Through the budding of the metula cells the secondary sterigmata layer composed of

uninucleate phialide cells is formed (Figure 1). Each of the phialide cells starts to produce uninucleate conidiospores, by subsequent repetition of budding. As a result, chains of conidiospores are formed from the phialide cells (Figure 1). Germination of a conidiospore will result in a homokaryotic mycelium formation.

Sexual reproduction in homokaryotic mycelium

The homozygous sexual cycle of *A. nidulans* begins in 4 days old colonies when the environmental conditions support the sexual development (darkness, decrease of oxygen tension, availability of ammonium nitrogen source). The first event of sexual development is the formation of unicellular Hülle cells (Figure 1). Hülle cells are thick walled multinucleate cells, which protect and nurse the developing asci. Surrounded by the Hülle cells, dikaryotic ascogenous hyphae are differentiated (Figure 1). These ascogenous hyphae form a ball shape nest, which is called primordium (Figure 1). Around the ball of ascogenous hyphae flattened longitudional hyphae are differentiated to the pericarp (outer coat/wall) of the immature closed fruiting body (cleistothecium). The primordium grows and forms the µ-cleistothecium, in which the ascogenous hyphae start to develop ascus mother cells and asci, where meiotic events occur (Figure 1). Upon maturation, the µ-cleistothecium hardens its pericarp that is accompanied by a dark pigment accumulation in the pericarp cells, while hundred thousands of asci are formed inside the cleistothecium (Figure 1). Each ascus contains eight binucleate, parental type ascospores with dark red color (Figure 1). When the free ascospores are released to the environment they germinate and form a homokaryotic mycelium.

Sexual, asexual and parasexual reproduction in heterokaryotic mycelium

When the mycelia of two neighbouring colonies are mixed, hypha anastomosis (plasmogamy) between the hyphae of different partners will occur from time to time (Figure 2). Following the anastomosis, the hyphal compartments are heterokaryotic, because they carry both types of nuclei originated from the two parental hyphae. The heterokaryotic compartments form the heterokaryotic mycelium. In nature as well as in the laboratory, the heterokaryotic mycelia are maintained when the environmental conditions are changed and the new conditions do not support the growth of the parental types of mycelia. If the strains carry complementary recessive genetic markers, the selection for heterokaryons can be easily achieved.

For example, a riboflavin auxotroph parent and a pyridoxine auxotroph parent are able to grow on a complete medium, which contains both the riboflavin and the pyridoxine vitamins. When mycelia of the two parental strains meet each other, anastomosis will occur between them and heterokaryotic compartments will be formed. However, the heterokaryotic state in these compartments won't be long lived, and the two parental nuclei will be separated in the following compartments and the homokaryotic state of the compartments will be restored. Completely different scenario takes place if a portion of the mixed mycelia (mixture of the two parental mycelia) is replaced to a minimal medium, which is free from

riboflavin and pyridoxine. In the absence of the required vitamins, only those compartments can grow and maintained, which are heterokaryotic, thus they contain both types of parental nuclei. In the heterokaryons the auxotrophies of the individual nuclei are complemented by the other parental nuclei. In such a selective environment, the homokaryotic mycelia will die, while the heterokaryotic mycelia thrive.

In the heterokaryotic mycelium sexual, asexual and parasexual life cycles may take place (Figure 2). Technically the the series of events are the same that happen during the homokaryotic conidiogenesis or sexual development, however the outcomes of these processes are different (Figure 2). In the heterokaryotic mycelium the dikaryotic ascogenous hyphae may contain two identical nuclei (homozygous dikaryon) of either parent or two different nuclei of the two parents

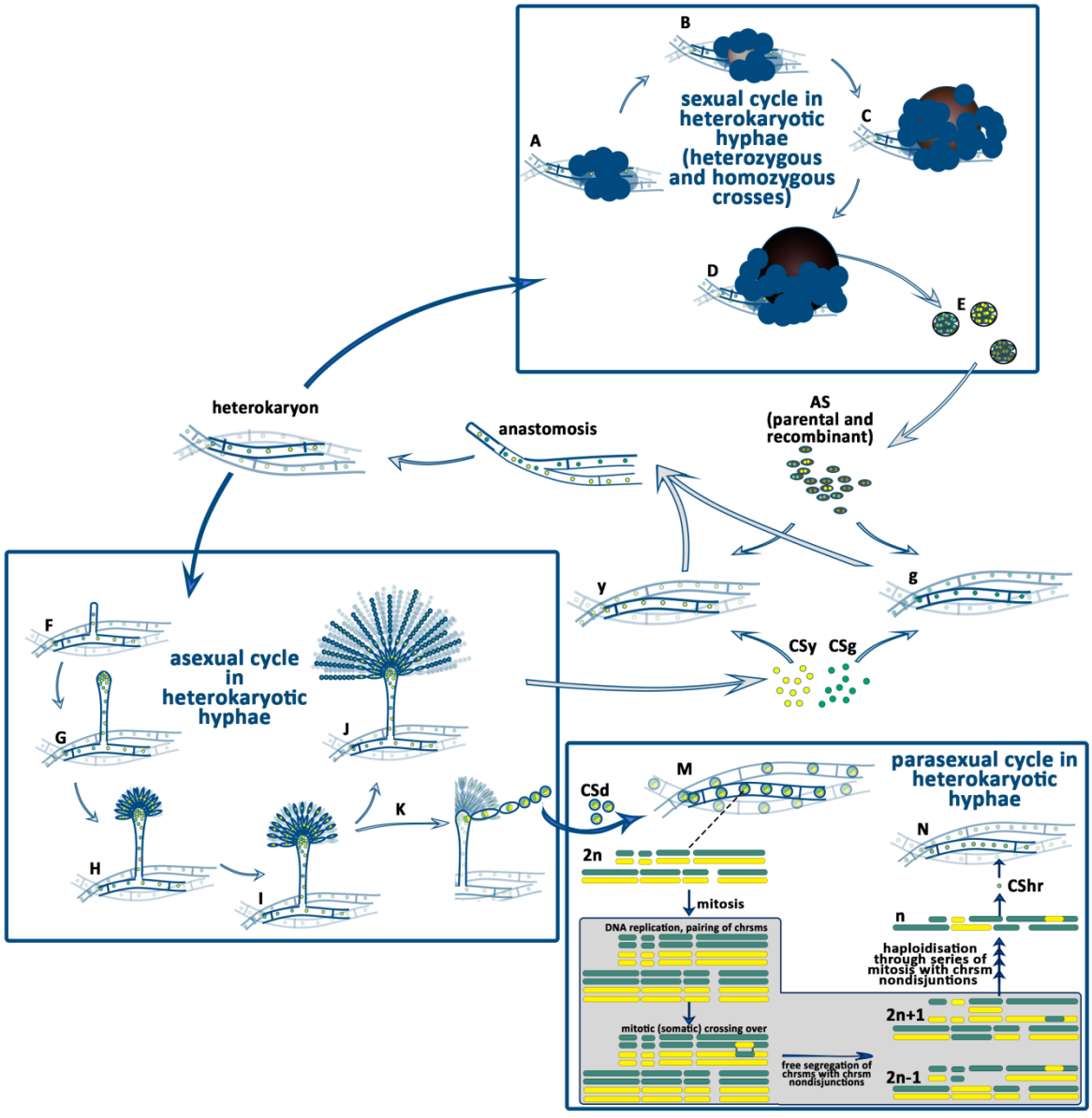

Figure 2. Life cycle of *A. nidulans* heterokaryotic hyphae.
<u>The heterozygous sexual cycle of *A. nidulans* (A-E)</u> begins with the production of Hülle cells and continues with the primordium (B) and μ-cleistothecium (C) formation as it is detailed in the homokaryotic sexual cycle in Figure 1. Upon maturation of the cleistothecia hundred thousands of asci are formed inside the cleistothecium (D). In case the ascogenous hypha in the primordium was homokaryotic, the whole cleistothecia will be of parental-type (D), thus all the ascospores inside will be

genetically identical with either the one or the other parent. In case the ascogenous hypha in the primordium was heterokaryotic, the whole cleistothecia will be of recombinant type (D). Germination of an ascospore will result in a homokaryotic mycelium formation. The heterozygous asexual cycle (the conidiogenesis) starts with the differentiation of a hyphal compartment into a foot cell and continues with the stalk (F), vesicle (G), metula layer (H) and the phialide layer (I) formation as it is decribed in details in the homokaryotic life cycle in Figure 1. Since the vesicle contains the nuclei of both parents and the metula cells formed by the mitosis of an individual nucleus of the vesicle are uninucleate, henceforth the metula cells may possess either one or the other parental nucleus. The uninucleate phialide inherits the nucleus of the underneath metula cell and the nucleus of the conidiospore will be identical with that of the phialid cell. Since one phialide cell produces many conidiospores that stick together forming a chain of spores, all the conidiospores belonging to the same chain will carry identical nuclei. Sometimes diploid nuclei are formed in the heterokaryon by the fusion of two haploid nuclei (K). When a heterozygous diploid nucleus plays role in metula cell formation, the metula cell and the subsequently formed phialide cell and all the conidiospores (CSd) in the cognate spore chain will carry a single diploid nucleus Germination of a diploid conidiospore will result in the formation of a diploid mycelium (M). When heterozygous diploid nuclei undergo mitosis, the failure of the segregation of sister chromatids, the so called chromosome non-disjunction, will result in the loss of one or more of the chromosomes in the progeny (grey boxed area). In these progeny the chromosome set is different from 2n and n (grey boxed area). They are called aneuploids. Over several mitoses the repeated events of chromosome non-disjunction will result in the haploidization of the diploid nuclei. The random losses of the parental chromosomes due to the chromosome non-disjunctions and the rarely occurring mitotic crossing overs will result in haploid genomes (n) that carry recombinant genetic material. When such a haploid and recombinant nucleus participates in conidiogenesis, the haploid and recombinant nucleus will be inherited in the conidiospores (CShr) and will be propagated in the environment. From these haploid and recombinant conidiospores a new, recombinant type of mycelia will be developed.

(heterozygous dikaryon). When the ascogenous hypha is homozygous, the ascospores in the whole cleistothecium will have the same parental nuclei (Figure 2). In case the ascogenous hypha is heterozygous, then the ascospores in the whole cleistothecium will have recombinant nuclei (Figure 2). Regarding the asexual reproduction, it will result in conidiosores with nuclei of either the one or the other parent. Nuclei of conidiospores belonging to the same chain will be always identical with each other, since they derive from the same uninucleate phialide cell. In the heterokaryotic mycelium heterozygous diploid nuclei may be formed by the fusion of two non-identical nuclei. Diploid formation is a relatively rare event (with 10^{-5}-10^{-8} frequency). The diploid nuclei will undergo mitosis and can take part in conidiogenesis that results in the formation of diploid conidiospores (Figure 2). The diploid conidiospores can germinate and form diploid mycelia. Sometimes the sister chromatides cannot separate from each other (called as chromosome non-disjunction) during the mitosis of the diploid nuclei and one nucleus will inherit both chromatides (it will possess three of that type of chromosome, the chromosome set will be 2n+1), while the other nucleus will not inherit that chromatid at all (it will posses only one from that type of chromosome, the chromosome set will be 2n-1) (see Figure 2). As a result of the chromosome non-disjunction, the chromosome set of the offsprings will be different from the haploid (n) and the diploid (2n), tehrefore the offsprings are called aneuploids. Repeated events of chromosome non-disjunctions lead to the haploidization of the genome, which may be visually detected in a diploid mycelium as the appearance of sectors. Due to the random losses of the parental chromosomes and the rarely occurring mitotic crossing overs (with 10^{-3} frequency) the

haploid genomes derived from the haploidization of a diploid genome will be of recombinant-type (differs from both parents). In the laboratory, the failure of the segregation of the sister chromatides, the chromosome non-disjunction can be mediated by the usage of the inhibitor of the microtubules, benomyl.

Literature:

Pontecorvo G. (1956) The parasexual cycle in fungi. Annu. Rev. Microbiol. 10, 393-100.

Pontecorvo G., Roper J. A., Hemmons L. M., Macdonald K. D., and Bufton A. W. J. (1953) The genetics of *Aspergillus nidulans*. Adv. Genet. 5, 141-238.

Kafer E. (1977) Meiotic and Mitotic Recombination in *Aspergillus* and Its Chromosomal Aberrations. In Advances in Genetics. Volume 19, Pages 33-131

3.1.3.1. Asexual life cycle of *A. nidulans*
- Practice 13 -

I. Introduction
- *Asexual life cycle of A. nidulans*

II. Practice 13
- Study of the asexual reproductive structures of *A. nidulans*

Learning Objectives

After the completion of the practical course, you should be able to:

- **Explain the different stages of conidiogenesis and name the structural units of a conidia.**
- **Define the following terms: uninucleate, conidiogenesis, metula, phialide, conidiospore**
- **Acquire the following laboratory skills: usage of the microscope, making cellux-tape preparates from conidia, calculate the necessay amounts of components of a laboratory used buffer.**

I. Introduction

Asexual life cycle of A. nidulans

When a uninucleate haploid conidiospore germinates, a colony will be formed by the mycelia of colorless septate hyphae (Figure 1). The compartments bordered by two septa are multinucleate and homokaryotic (Figure 1). 40-48 hour after the germination of a conidiospore, the young colony starts to develop foot cells by the differentiation of certain compartments (Figure 1). A foot cell gain a brownish thick cuticle and produces an up-growing stalk (conidiophore) with length of 100 µm and diameter of 6 µm (Figure 1). At the end of the stalk a vesicle is formed, 10 µm in diameter (Figure 1). The foot cell, the stalk and vesicle are multinucleate, aseptate and covered with a thick brownish cuticle. From the surface of the vesicle uninucleate elongated buds with 5 µm in length are developed synchronously (Figure 1) by budding. These daughter cells are called metula cells and they form the primary sterigmata layer of the conidia. The metula cells will undergo a budding process synchronously, giving origin to a layer of uninucleate phialide cells (Figure 1). This second layer of cells is called secondary sterigmata layer. Each phialide cell begins a series of budding, by which a chain of uninucleate conidiospores will emerge from the end of the phialide cell (Figure 1). The youngest conidiospore is always the nearest one to the phialide cell. The older ones are further away, gradually attaining full size and full green color upon maturation. All the conidiospores of a certain chain derive their nucleus from the cognate single nucleus of the sterigma cells (phialide and metula). The conidiospores are green in wild type *A. nidulans*.

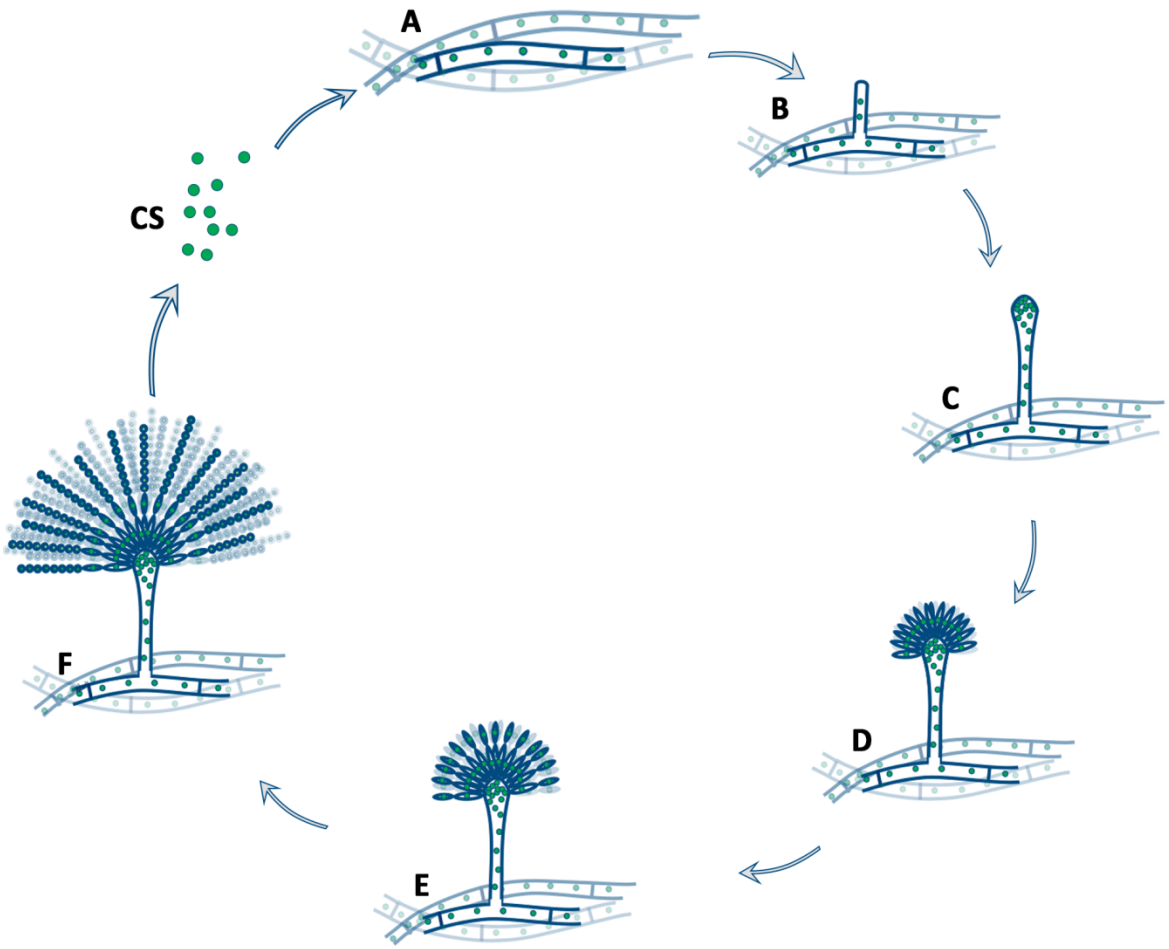

Figure 1. Conidiogenesis.
Conidiogenesis starts with the differentiation of a vegetative hyphal compartment (A, B) by the development of the stalk high above the surface of the colony (B). At the end of the stalk a vesicle containing many nuclei is formed (C). Nuclei in the vesicle undergo mitosis and by budding (same as budding in yeasts) a layer of uninucleate cells (primary sterigmata layer) bud out of the vesicle (D). These daughter cells are called metula cells. After that the metula cells will synchronously undergo budding and as a result, a secondary sterigmata layer of uninucleate phialide cells are formed (E). After that the phialide cells begin a series of budding, by which chains of conidiospores are formed at the end of the phialide cells. During maturation, the conidiospores are kept together in the chain. Upon maturing, the conidiospores are disbanded and will be spread as free individual conidiospores by the air or water to new habitat.

Literature:

Pontecorvo G., Roper J. A., Hemmons L. M., Macdonald K. D., and Bufton A. W. J. (1953) The genetics of *Aspergillus nidulans*. Adv. Genet. 5, 141-238.

II. Practice 13

Goal

Study of the asexual reproductive structures of *A. nidulans* in light microscope.

Required materials

Strain: HZS.145 (*veA1*)

One Petri dish of 3 days old HZS.145 culture grown on CM at 37 °C. Cellux tape; scissors; slide; cover slips; 20-200 µl pipette and tips; 1 x PBS (phosphate buffered saline pH 7.4; 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4); light microscope.

Tasks

1. Calculate the amount of the components needed for the preparation of 150 ml 1 x PBS!

The molecule weights of the PBS components are the following:

1 M NaCl Mw: 58.4 g

1 M KCl Mw: 74.551 g

1 M Na_2HPO_4 Mw: 141.96 g

1 M KH_2PO_4 Mw: 136.086 g

Amounts of the components needed for the preparation of 150 ml 1 x PBS:

NaCl: .....

KCl: .....

Na_2HPO_4: .....

KH_2PO_4: .....

2. Take conidia samples from the colonies by touching the sticky side of the cellux tape to the surface of a colony and mounting it to 1 drop of PBS placed on the slide. Eliminate the unnecessary parts of the cellux tape, then add a drop of PBS to the surface of the cellux tape and cover the sample with a cover slip. Study the structures of the conidia by using 20 x and 40 x objectives!

Make a draw of the conidia and name its parts!

Check your progress

After the completion of the practical course, answer the following questions.

- **What does it mean:**
  - conidia
  - conidiospore
  - foot cell
  - stalk
  - vesicle
  - metula cell
  - phialide cell?
- **Why do we use 1 x PBS solution for mounting of biological samples?**
- **You need to prepare 200 ml 10 x TE buffer (10 mM Tris/HCl, 1 mM EDTA pH 8.0) in the laboratory. How many gramms of the ingredients do you put in 200 ml volume?**

3.1.3.2. Formation and maintenance of heterokaryons - Practice 14 -

I. Introduction

- *Heterokaryon formation in nature*
- *Introduction to the concept of balanced and unbalanced heterokaryons and Discrimination between balanced and unbalanced heterokaryons in practice*
- *Introduction to heterokaryon incompatibility in nature*
- *Introduction to the concept of protoplast formation and fusion of protoplasts for obtaining heterokaryons*

II. Overview of the course

II. Practice 14 – from week 1 to week 3

- 1st week
- 2nd week
- 3rd week

Learning Objectives

After the completion of the practical course, you should be able to:

- **Understand the concept of heterokaryon formation and execute heterokaryon formation in practice**
- **Understand the concept of the maintenance of heterokaryons and gain experimental skill for the maintenance of heterokaryons**
- **Define the following terms: homokaryon, heterokaryon, anastomosis, protoplast, spheroplast, heterokaryon incompatibility, uninucleate, conidiogenesis, metula, phialide, conidiospore**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, learning sterilization techniques, usage of automatic pipettes, usage of sterile cabinets, preparation of culture media, inoculation of filamentous fungi, culturing of filamentous fungi on solid media, portoplast formation from the filamentous fungus *Aspergillus nidulans*, obtaining and maintenance of naturally formed heterokaryons, obtaining heterokaryons by protoplast fusion**

Heterokaryon formation in nature

Heterokaryons are formed naturally by anastomosis of hyphae growing close proximity to each other (Figure 1). In case the fusion occurs between two isogenic hyphae, the result is the formation of a homokaryotic hypha. When hyphae of two different mutants fuse together, the result is the formation of heterokaryotic hypha, which will form heterokaryotic mycelia (heterokaryon) upon growing. When a mixed colony of two different auxotrophic strains (e.g. a riboflavin auxotroph *riboB2* strain and a para-amino-benzoic acid auxotroph *pabaA1* strain) is transferred to a minimal medium, which is not supplemented with neither riboflavin nor para-amino-benzoic acid, only the heterokaryotic hyphae can grow, where the two different auxotrophic nuclei complement each other's auxotrophy. Under the selection pressure provided by the minimal medium free from riboflavin and para-amino-benzoic acid, the growing heterokaryotic hyphae will form a heterokaryotic colony with heterozygous conidia (Figure 1).

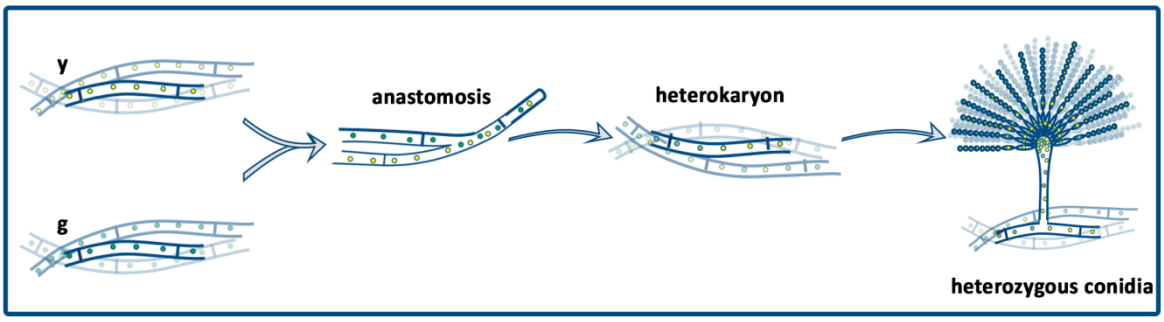

Figure 1. Formation of heterokaryotic mycelia and heterozygous conidia.
y: hyphae of parent 1 marked with yellow nuclei; g: hyphae of parent 2 marked with with green nuclei.

Since mitosis of individual nuclei located in the vesiculum via budding will form the primary sterigmata layer (layer of metulae) during the process of conidiogenesis, each metula cell will contain a single nucleus (Figure 2 of General Introduction section of 3.1.3.). Therefore, the phialides, which are formed by the budding of metula cells will also carry a single nucleus and the conidiospores generated by the budding of phialide cells will be uninucleate, too (Figure 2 of General Introduction section of 3.1.3.). As a consequence of the uninucleate nature of the conidiospores, the heterokaryotic state fromed by two auxotrophic strains cannot be maintained by the inoculation of conidiospores. Heterokaryons can be passed from one minimal medium to

other plate with minimal medium by inoculating the heterokaryotic mycelia, not the conidiospores. Conidiospores from heterokaryons will never germinate on the minimal medium.

Introduction to the concept of balanced and unbalanced heterokaryons and Discrimination between balanced and unbalanced heterokaryons in practice

A heterokaryon is balanced when the two types of nuclei are equally represented in the heterokaryon colony. When one nucleus is overrepresented, then we say that the heterokaryon is unbalanced. Unbalanced heterokaryon is formed when one type of nucleus divides mitotically more rapidly in comparison to the other type of nucleus in the heterokaryon. Unbalanced heterokaryons might indicate that a mutation affects the mitotic process or the general fitness of the fungus. We can assess the balance of the heterokaryon easily by using auxotrophic strains with different conidiospore colors (*e.g*. green and white conidiospore colors) for the heterokaryon formation. To estimate the balance of the heterokaryon conidiospores must be collected from a 1 cm^2 region of the heterokaryon, stepwise series of dilution must be made from the conidiospore suspension followed by the spreading of an aliquot to complete medium from the different steps of conidiospore dilutions and counting the number of the white and green colonies after incubation.

Introduction to heterokaryon incompatibility in nature

In case of some filamentous fungi, such as imperfect black Aspergilli, two strains belonging to the same species might be heterokaryon incompatible (vegetative incompatible). It means that in nature the hyphae of incompatible strains will never fuse. In some cases however, in the laboratory, we can force heterokaryon formation between incompatible strains by obtaining protoplasts via treating the mycelia with wall-digesting enzyme mix followed by the forced fusion of the cell membranes by adding PEG-4000 (polyethylen glycol-4000) into the protoplast mixture of two strains.

Introduction to the concept of protoplast formation and fusion of protoplasts for obtaining heterokaryons

The term protoplast is a technical phrase for a cell, which lost its cell wall. When the cell is not completely naked but partially covered with cell wall, we call it a spheroplast. In order to obtain a successful fusion between two strains we must eliminate the cell wall completely, thus obtaining perfect protoplasts. For the elimination of the cell wall of fungi we use enzyme coctails, which are able to digest the covalent bounds between cell wall components. For *Aspergillus* species cell wall lysing enzyme purified from *Trichoderma sp.* (Glucanex), rich in β-glucanase, cellulase, protease and chitinase is used for protoplast formation. After forcing cell membrane fusions between protoplasts by applying polyethylene glycol-4000 (PEG-4000) treatment, the fused protoplasts are regenerated on selective minimal medium. By using different parental selection markers, we may select for those regenerated fusions, where nuclei of two parental strains shares the same cytoplasm.

Literature:

Pontecorvo G. (1956) The parasexual cycle in fungi. Annu. Rev. Microbiol. 10, 393-100.

Pontecorvo G., Roper J. A., Hemmons L. M., Macdonald K. D., and Bufton A. W. J. (1953) The genetics of *Aspergillus nidulans*. Adv. Genet. 5, 141-238.

II. Overview of the course

1. Inoculation of the two parental strains HZS.119 and HZS.544 into the surface of cellophane-covered CM and regular CM in alternated pattern for protoplast fusion and naturally formed heterokaryon isolation, respectively.

2. Protoplast formation by glucanex treatment of parental colonies on the surface of cellophane sheets and fusion of the parental protoplasts by PEG-4000 treatment. Cutting out agar disks from the alternately growing colonies from those regions where the two parental colonies contacts physically. Pouring top agar containing fused protoplasts into vitamin and methionine free minimal medium, and placing the colony disks (cut from the alternately grown colonies) onto the surface of vitamin and methionine free minimal medium

3. Observation of heterokaryons obtained by protoplast fusion method and by selection for naturally formed heterokaryons. Visual estimation of the balanced status of the heterokaryons by comparison of the ratio of yellow and white conidiospores.

III. Practice 14 – from week 1 to week 3

1st week

Goal

Inoculation of the two parental strains HZS.119 and HZS.544 to the surface on CM cellophane plates for protoplast formation. Inoculation of the same parental strains to CM in alternate pattern (see Figure 2) for obtaining naturally formed heterokaryons

Required materials

Used *A. nidulans* strains: HZS.119 (*yA2,anA1, riboB2, veA1*)
HZS.544 (*wA3, pabaA1, veA1*)

Required materials: Three days old culture of *A. nidulans* strains HZS.119 and HZS.544 grown on complete medium (CM) (2% (v/v) salt solution, 1% (w/v) glucose, 2 g/l pepton, 1.5 g/l casamino acids, 1 g/l yeast extract, 2.5% (w/v) agar, pH 6.8 supplemented with multi-vitamin). Seven freshly prepared, wet Petri dishes with CM. Six sterile cellophane disks with 9 cm diameter. Two sterile paint-brush, forceps, 2 sterile glass test-tubes with aluminium caps filled with 1 ml 0.01% Tween-80, loop inoculator, 4 sterile Petri dishes with 9.5 cm diameter,

Tasks

Place one sterile cellophane disk on the surface of feach reshly prepared, still wet Petri dishes containing CM using the sterile cabinet. Use the forceps to evenly spread the cellophane sheets and eliminate air bubbles squeezed between the medium and the cellophane sheet. Prepare conidiospore suspension in 1 ml Tween-80 from strains HZS.119 and HZS.544 by loop inoculator. By using the paint-brushes and the conidiospore suspension, inoculate 3 cellophane covered

plates with strain HZS.119 and 3 cellophane covered plates with strain HZS.544. During the paint-brush inoculation submerge the tip of the paint-brush into the conidiospore suspension, squeeze the liquid from the paint-brush by pushing the brush to the dry inner surface of the glass test-tube. The still wet hair of the paint-brush will carry thousands of conidiospores. Now gently touch the surface of cellophane 5-8 times in different spots and after that take new sample by submerging again the paint-brush in the conidiospore suspension. Repeat the inoculation until you create 50-60 inoculation spots all over the surface of the cellophane. Place the inoculated cellophane plates in 37 °C incubator overnight.

Dry one CM dish and make spot inoculations in alternate pattern (Figure 2) using the loop inoculator and your conidiospore suspension of 1107 and 1110 strains. Place the inoculated cross plate in 37 °C incubator.

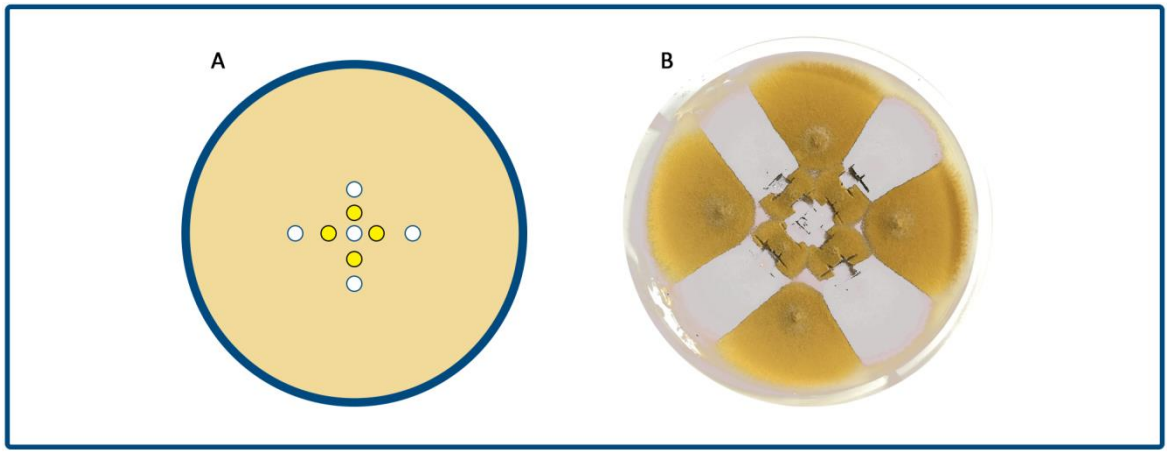

Figure 2: Alterante pattern inoculation (panel A) and cut of agar disks at regions where the two parental strains established physical contact (panel B).

Goal

1. Isolation of naturally formed heterokaryon by cutting out agar disks from the cross plate from regions where the two parental strains established physical contact. Transfering the disks to a new plate with minimal media.
2. Obtaining protoplasts from the cellophane cultures and inducing fusion between protoplasts of the two parental strains followed by their inoculation to minimal media for isolation of heterokaryons.

Required materials

Overnight cellophane cultures of strains HZS.119 and HZS.544. Sterile scalpels, spear point needle, one small Petri dish filled with MM (2% (v/v) salt solution, 1% (w/v) glucose, 10 mM $NaNO_3$, 2.5% (w/v) agar, pH 6.8), cellux tape, forceps, 100 mg glucanex enzyme, 200 ml 0.7 M KCl, 2 sterile empty Petri dishes, 2 sheets of sterile cheese filters with 100 µm pore diameter, 2 sterile glasses with 500 ml capacity, 6 sterile cellophane capped glass centrifuge tubes with 25-30 ml capacity, 2 ml eppendorf tubes (at least 3 pieces), 0.2-1 ml pipette and tips, 0.02-0.2 ml pipette and tips, 12 aluminium capped glass test tubes with 4.5 ml 0.7 M KCl, TN1 solution (for 100 ml TN1: 5.22 g 0.7 M KCl, 0.735 g 50 mM $CaCl_2$), freshly prepared TN2 solution (for 5 ml TN2: 250 µl 1 M $CaCl_2$, 500 µl Tris/HCl, 3g PEG-4000), 50 ml freshly prepared MM top agar kept at 42 °C degree (MM with 1% agar), 100 ml CM top agar kept at 42 °C degree (CM with 1% agar), 6 Petri dishes with selective sucrose medium (SMM) (2% (v/v) salt solution, 1% (w/v) glucose, 10 mM $NaNO_3$, 1 M sucrose, 2.5% (w/v) agar, pH 6.8), 18 Petri dishes with CM supplemented with 1 M sucrose (SCM), water bath with 42 °C degree, one sterile 15 ml Falcon tube, 24 sterile 50 ml falcon tube or 20 cm long sterile glass test tubes with aluminium caps, microscope, haemocytometer (Burker chamber).

Tasks

1. Cut out agar disks from the cross plate from regions where the two parental strains established physical contact and transfer the disks face down to a new plate with minimal media.

2. Peel off the cellopane sheets with HZS.119 and HZS.544 cultures and place the three sheets into a single empty Petri dish. Soak the cellophane sheets in 5 ml freshly prepared glucanex solution (5 ml glucanex solution/plate, 10 mg glucanex / 1 ml of 0.7 M KCl) and incubate them for about 1 hour 15 minutes at room temperature. Monitor the protoplast formation in microscope. When protoplast formation is completed, wash the cellophane sheets in 100 ml 0.7 M KCl and filter the protoplast suspension through a cheese filter with 100 µm large pores. Collect the protoplasts by centrifugation at 4000 g for 25 minutes at 14 °C. Discard the supernatant and suspend the pellet by hand shaking (do not vortex). Wash the protoplasts with 10 ml of 0.7 M KCl and after collecting them by centrifugation (4000 g for 25 minutes at 14 °C) resuspend them in 1 ml 0.7 M KCl. Count the protoplasts in a haemocytometer (Burker chamber) under a light microscope. Document the calculated numbers of ptoroplasts. In order to determine the protoplast regeneration efficiency of the parental protoplasts, prepare stepwise ten fold dilution series from the protoplast samples of HZS.119 and HZS.544 by taking out and adding 0.5 ml protoplast samples to 4.5 ml 0.7 M KCl (dilution 10^{-1}). After the consecutive repetition of this ten fold dilution process take out 1 ml samples from the last two steps of the series (dilutions 10^{-3} and 10^{-4}), place them into 50 ml Falcon tubes (or 20 cm long glass test tubes) and add 15 ml SCM top agar to them. Shake and evenly distribute the mixture on the surface of three plates of SCM sucrose medium. For protoplast fusion, mix $5x10^{5}$ protoplasts of strain HZS.119 and HZS.544 into a single eppendorf tube and centrifuge the mixed protoplasts at 2500 rpm for 6 minutes. Discard the supernatant and suspend the protoplasts in 200 µl TN1 solution. Add 1 ml of TN2 solution to the sample and transfer the sample into a 15 ml large Falcon tube and add 10 ml KCl to it. Centrifuge the protoplasts at 4000g for 20 minutes at 14 °C. Suspend the protoplasts into 1 ml 0.7 M KCl by hand shaking. Prepare stepwise dilution series of the fused protoplasts by using 0.5 ml of protoplast sample and the glass test tubes with 4.5 ml 0.7 M KCl. In order to determine the regeneration efficiency after fusion, transfer 1 ml of the last two dilutions of the series (dilutions 10^{-3} and 10^{-4}) to 50 ml Falcon tubes (or 20 cm long glass test tubes) and add 15 ml freshly prepared SCM top agar (40-42 °C) to each sample. Mix the protoplast samples with the top agar by hand shaking and distribute the top agar on the suface of three SCM plates (about 5 ml/petri dish) in each case. Try to spread the top agar on the surface of SCM medium evenly by gently moving the plates. In order to isolate heterokaryons transfer 1 ml of the last two dilutions of the series (dilutions 10^{-3} and 10^{-4}) to 50 ml Falcon tubes (or 20 cm long glass test tubes) and add 15

ml freshly prepared SMM top agar (40-42 °C) to each sample. Mix the protoplast samples with the SMM top agar by hand shaking and distribute the top agar on the suface of three SMM plates (about 5 ml/petri dish) in each case. Incubate the plates for 4-5 days at 37 °C.

Goal

Investigation of the heterokaryons formed naturally and by using protoplast fusion method in stereomicroscope. Estimation of the ratio of yellow and white conidiospores in the heterokaryon and assessment of balanced status of the heterokaryon. Calculation of the protoplast regeneration frequency of parental strains and the regeneration frequency of protoplasts after the fusion by counting CFUs (Colony Forming Units) on the parental and fusion regeneration control SCM plates. Monitor the colonies for conidiospores with green color, which are diploids.

Required materials

Stereomicroscope, SMM plates of heterokaryons formed naturally and by protoplast fusion and SCM plates of parental and fusion regeneration controls.

Tasks

Place the plates of heterokaryons formed naturally and by protoplast fusion under stereomicroscope and study the color of conidiospores. Try to estimate the ratio of the white and the yellow colored conidiospores in order to estimate whether you have balanced or unbalanced heterokaryons. Count the CFUs on the parental and fusion regeneration control plates. Determine the regeneration frequency (in %) of parental protoplasts and fused protoplasts on the basis of the counted CFUs, the protoplast concentration (calculated from Burker chamber counts during the previous week) and the applied dilutions.

Results:

Heterokaryon: balanced unbalanced

Regeneration frequency of parental strain 1107: .....%

Regeneration frequency of parental strain 1110: .....%

Regeneration frequency of fused protoplasts: .....%

Check your progress

After the completion of the practical course, answer the following questions.

- **What does it mean:**
  - **protoplast**
  - **spheroplast**
  - **protoplast regeneration frequency**
  - **heterozygous dikaryon**
  - **homozygous dikaryon**
  - **homokaryon**
  - **heterokaryon**
  - **anastomosis**
  - **karyogamy**
  - **heterozygotic cross**
  - **homozygotic cross?**
- **What is the role of 0.7 M KCl as supplement in all those solutions, in which protoplasts are formed or resuspended?**
- **Why the protoplast regeneration media do not supplemented with 0.7 M KCl?**
- **Why complete medium and not minimal medium is used for the plating of parental protoplasts or fused protoplasts for the purpose of determining the protoplast regeneration frequency?**
- **What is the explanation for that distinct chains of conidiospores budded from single phialides are always composed from conidiospores with the same color (in the chain the colors never appear mixed)?**

2.1.3.3. Sexual life cycle of *Aspergillus nidulans* - Practice 15 -

I. Introduction

- Life cycle of *Aspergillus nidulans*
- *Description of the velvet (veA1) strain*
- *Terminology: auxotroph and prototroph strains*
- *Genetic cross between two genotypes in practice*
- *How to write and read genotype? – The nomenclature of A. nidulans*
- *Color of the conidiospores*
- *Vitamin auxotrophy used during the course*
- *Literature*

II. Overview of the course

III. Practice 15 – from week 1 to week 7

- 1st week
- 2nd week
- 3rd week
- 4th week
- 5th week
- 6th week
- 7th week

Learning Objectives

After the completion of the practical course, you should be able to:

- **Describe the sexual life cycle of *A. nidulans***
- **Understand the concept of genetic cross between two *A. nidulans* strains**
- **Describe briefly the execution of a genetic cross between two *A. nidulans* strains**
- **Describe the role of VeA protein in the light-dependent regulation of sexual development**
- **Describe the role of CO_2 in the initiation of sexual development of *A. nidulans***
- **Explain the genetic background of the formation of green pigment in conidiospores**
- **Read out and explain *A. nidulans* genotype descriptions**
- **Explain the concept of progeny analysis after genetic cross**
- **Define the following terms: homothallic, mating type determining factors, self-fertile, cleistothecia, µ-cleistothecia, homokaryon, heterokaryon, dikaryion, anastomosis, karyogamy, differentiation, Hülle cell, ascogenous hypha, peridium, ascus mother cell, hook formation, ascus, ascospore, uninucleate, conidiogenesis, nuclear localisation signal, parental, recombinant, heterozygotic cross, homozygotic cross, heterozygous dikaryon, homozygous dikaryon**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, learning sterilization techniques, usage of automatic pipettes, usage of sterile cabinets, preparation of culture media, usage of stereomicroscope, inoculation of filamentous fungi, culturing of filamentous fungi on solid media, genetic cross of *A. nidulans*, progeny analysis of a genetic cross**

I. Introduction

Sexual life cycle of Aspergillus nidulans

A. nidulans is a homothallic fungi, with each nuclei of the colony carrying and (upon activation) simultaneously expressing the mating type determining factors, the α-box protein (coded by *matB* gene) and the HMG-box protein (coded by *matA* gene). Due to its homothallic nature, the *A. nidulans* is self-fertile, therefore it is an excellent model organism for the study of the fungal sexual reproduction.

The sexual reproduction is largely governed by environmental factors. The balance of the available carbon- and nitrogen sources (Han et al., 2003), darkness (Yager, 1992), neutral pH (Rai et al., 1967) and the elevated level of CO_2* (Zonneveld, 1977) support the sexual cycle. This latter is commonly achieved in the laboratory by tightly sealing the culturing plates in order to limit air exchange. Zonneveld and co-workers showed that CO_2 is needed for both the synthesis and breakdown of α-1,3-glucan, which were found as pivotal factors for the production of fruiting bodies (cleistothecia) (Zonneveld, 1988). Sealing of the plates results in the decrease of the oxygen concentration, which causes the irreversible entry into the sexual cycle. Other environmental factors, such as stress factors (strong light, osmotic stress) inhibit the sexual cycle (Han 2009).

As the first step of the sexual cycle, two homokaryotic hyphae fuse into a heterokaryotic hypha (Figure 1.)

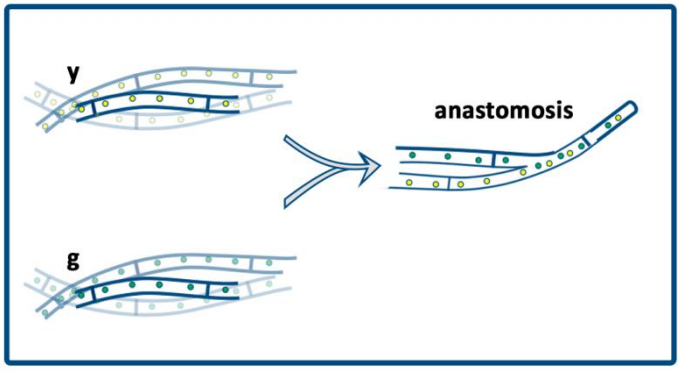

Figure 1: Formation of heterokaryon by hypha anastomosis.
y: hyphae of parent 1 marked with yellow nuclei; g: hyphae of parent 2 marked with with green nuclei.

* Elevated level of CO_2 represses sexual differentiation in other fungi, such as *Schizophyllum*, *Penicillium* and *Agaricus* (Sietsma et al., 1977, Graafmans, 1973, Long and Jacobs, 1974)

The sexual reproduction requires the co-ordinated differentiation of three cell-types. These are the Hülle cells (Sarikaya Bayram et al., 2010), ascogenous hypha and the cleistothecium wall (peridium/pericarp) forming flattened cells.

Macromorphologically the sexual reproduction involves the following stages. Amongst the net of hyphae, specialized, thick walled Hülle cells are produced. Hyphae in the neighbourhood of the grouping Hülle cells (h, Figure 2) form a nest, which is called primordium (p, Figure 2). Specialized flattened cells differentiate from the aerial hyphae and form the wall of the fruit body (first it is called pericarp – pc; later it is called cleistothecium wall – cw on Figure 2). Inside the maturing young cleistothecium (µ-cleistothecium) dikaryotic ascogenous hyphae are formed (ah, Figure 2) with asci (as, Figure 2). Each ascus will contain 8 non-ordered ascospores. The mature cleistothecium is a shiny dark-purple/blackish closed ball (Figure 2).

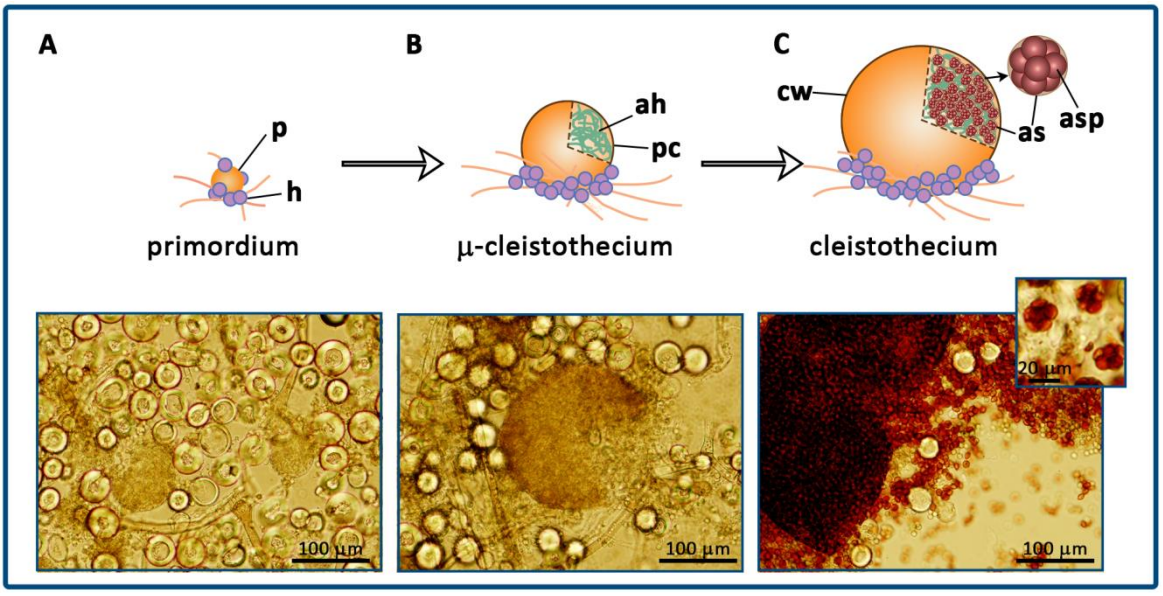

Figure 2: Structures of sexual development in *A. nidulans*.
A: Primordiums (p) surrounded by Hülle (h) cells. B: Primordiums develop into m-cleistothecia, which are composed from ascogenous hyphae (ah) inside and pericarp (pc) outside. C: Upon maturation the pericarp develops to cleistothecium wall (cw) and ascogenous hypha will produce and fill up the cleistothecia with asci (as). Each ascus contains eight ascospores (asp). Microscopic picture below the drawing shows the corresponding developmental stage. Microscopic pictures were done by Eszter Bokor.

Hülle cells are large (approx. 10–15 µm diameter), thick-walled single cells that are formed in large number (Figure 3). Hülle cells are multinucleate, although at maturity a big macronucleus (with a volume 20x than that of normal nuclei) is formed by the grouping of smaller nuclei (Carvalho et al., 2002). Hülle cells surround cleistothecia during development (Sarikaya Bayram et al., 2010) and due to their specialized physiology, (e.g. laccase and chitin synthase activity) (Bayram & Braus, 2011) they are able to 'nurse' the cleistothecia during development, such as through the production of α-1,3 glucanase that mobilizes carbon resources required for fruiting body development (Wei et al., 2001).

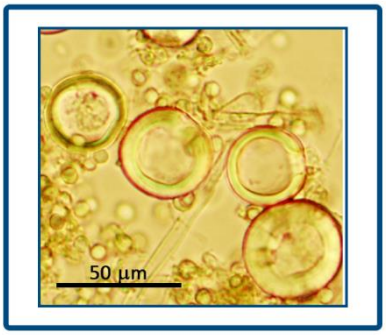

Figure 3: Thick-walled Hülle cells. Microscopic pictures were done by Eszter Bokor.

The peridium or pericarp is composed of two layers of darkly pigmented flattened cells, which are glued together (it is thought that a substance, called 'cleistin' glues them together) (Sohn & Yoon, 2002).

Inside the pericarp, sexually differentiated, dikaryotic ascogenous hyphae are found (Figure 2, picture in middle, Figure 4 and 5), which can be heterokaryotic as well as homokaryotic. The ascogenous hyphae have an irregular wavy shape (Figure 4). Karyogamy (heterokaryons in genetic cross, homokaryons in selfing) and hook formation take place in the compartments of the ascogenous hyphae by a process described in Figure 6.

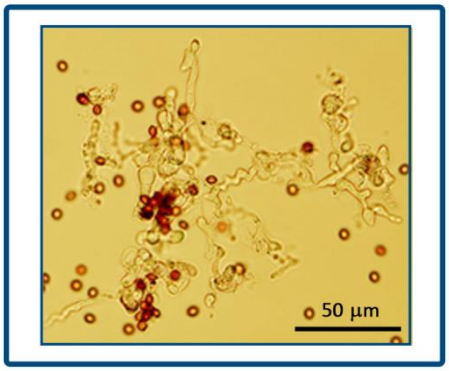

Figure 4: Wavy ascogenous hyphae with some red ascospores. Microscopic pictures were done by Eszter Bokor.

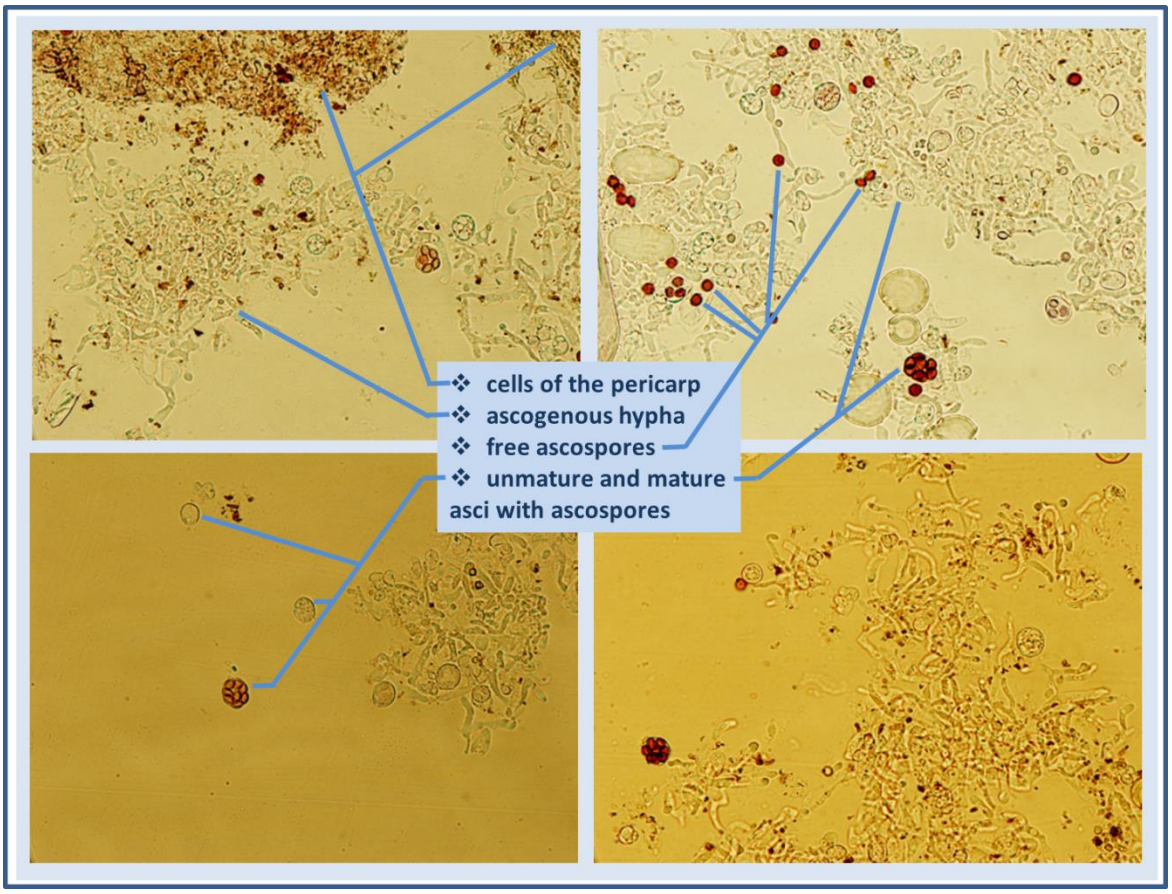

Figure 5: Sexual structures inside the cleistothecium. Microscopic pictures were done by Eszter Bokor.

The two nuclei in the apical compartment (can be heterokaryones or homokaryones) (A in Figure 6) undergo mitosis simultaneously (B in Figure 6). The four nuclei will be separated in space by forming three compartments. The compartment at the top contains two nuclei (C-E in Figure 6), which will fuse together (karyogamy) and a diploid zygote will be formed (F in Figure 6). This compartment is called ascus mother cell. The remaining two nuclei are separated from the others and form the basal (at the bottom of the ascus mother cell) and the middle compartments (at the side of the ascus mother cell) (C in Figure 6). The side compartment elongates and bends over to form a hook (crozier) (B-C, Figure 6). The elongation of the hook takes place until it reaches the uninucleate basal compartment, where the two uninucleate compartments fuse together by anastomosis (D in Figure 6). The produced dikaryotic compartment is equivalent with the dikaryotic cell shown at the beginning of the whole process (A in Figure 6) and undergoes mitosis (E in Figure 6). This step is followed by the separation of the four nuclei in three compartments (F in Figure 6), which means the formation of a new mother cell and a new hook. The whole process is repeated for several thousands times. When an ascus mother cell is formed, the karyogamy and diploid formation take place inside it (F in Figure 6). The diploid nucleus undergoes meiosis immediately (G in Figure 6) and as a result, four haploid nuclei are formed. Then each haploid nucleus divides mitotically, and as a result eight haploid nuclei are produced in

the young ascus (H in Figure 6). Cytokinesis takes place and the eight nuclei will be separated from each other by cell membranes and cell walls. The eight ascospores are formed. Unmature ascospores are uninucleate and free of pigments. Upon maturation, the nucleus of an ascospore goes through mitosis and a dark reddish pigment is accumulated in the cell wall. Henceforth the mature ascospores are binucleate and gain a dark red color.

In a mature cleistothecia up to 100.000 asci can be found, each enclosing eight ascospores. An average cleistothecium may contain around 80.000 viable ascospores (Pontecorvo, 1953; Braus et al., 2002).

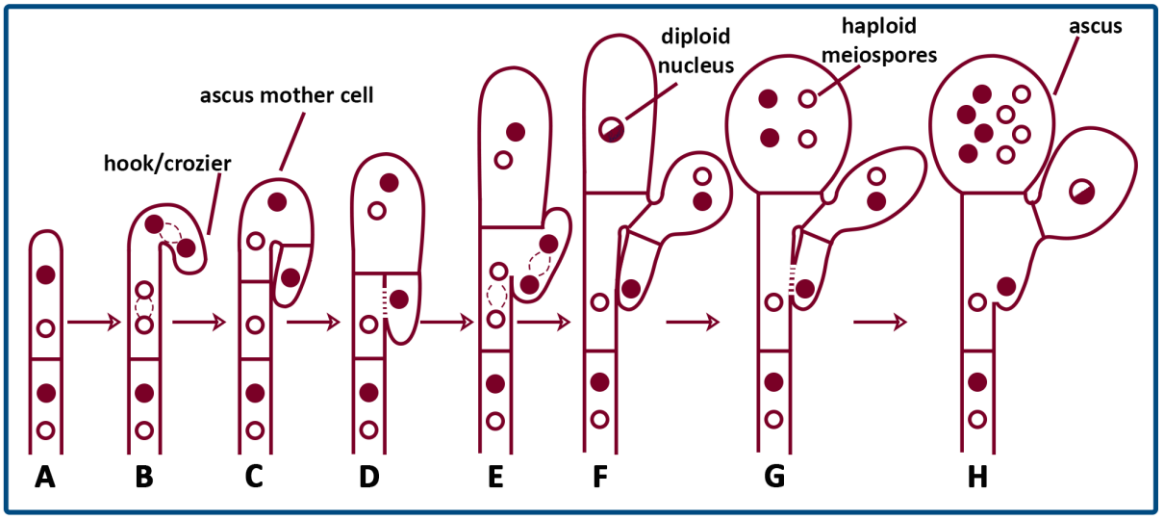

Figure 6: Stepwise schematic description of hook formation and meiotic events in the dikaryotic ascogenous hyphae. Dashed lines between nuclei marks tubulins of the spindle body. Filled and empty dots mark the two different parental types of nuclei.

Description of the velvet (veA1) strain

Regulation of sexual and asexual repoduction cycles and the secondary metabolite production overlaps in *A. nidulans* by shared regulatory elements belonging to the "velvet" protein family (VeA, VelB, VelC and VosA; Figure 5) and the histone methyl transferase LaeA (Bayram and Braus 2012).

Genetic work with *A. nidulans* as a model organism has been started in 1960's with the usage of an environmental strain, which had a particular mutant phenotype called velvet after its velvet-like surface (Figure 7) (Käfer 1965). The wild type strains produce conidiospores when the mycelia are exposed to light and sexual processes are initiated in dark. The wild type colonies are always richly covered with aerial hyphae, which makes hard to gain access to conidiospores and fruiting bodies on the colonies (Figure 7). What made the velvet mutant popular in the laboratory was that it produced aerial hyphae at reduced level, increased asexual sporulation and reduced

sexual development in the dark (Figure 7). Since the time of isolation of the velvet strain, nearly all of the laboratories (except those, which work on secondary metabolism and sexual development) work with the descendants of the original *veA1* strain.

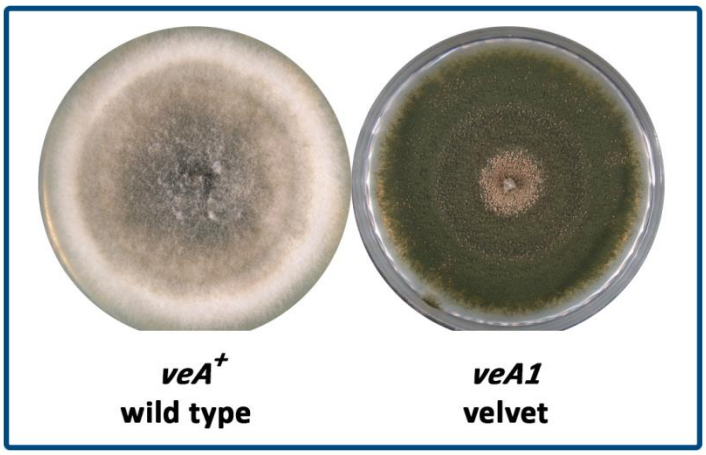

Figure 7: Colonies of wild-type (veA+) and veA1 A. nidulans. Photographs were done by Eszter Bokor.

Kim et al. (2002) revealed that the velvet strain carries a point mutation in the third nucleotide of the start codon of the *veA* gene (ATG is converted to ATT). Due to the mutation, the next available ATG is used for initiation, which corresponds to the 37th Methionine amino acid residue (37Met) of the wild type protein. Thus the *veA1* mutant produces a truncated version of the VeA protein, which lacks the first 36 amino acids. Since a nuclear localisation signal (NLS) is coded in these first 36 amino acid long region of the protein, the *veA1* mutant cannot enter the nucleus due to the NLS is missing. With the truncated VeA1 protein the mutant is able to complete the sexual development by an illumination-independent manner, and produces less amount of cleistothecia than the wild type (Kim et al. 2002). Since nuclear localization of VeA is important for the light-dependent regulation of sexual and asexual development, the regulation role of light is switched off in the *veA1* mutant, therefore they are able to form conidiospores in dark as well as in light and cleistothecia in light as well as in dark (Kim et al., 2002, Stinnett et al., 2007, Bayram et al., 2008). Additionally to the alteration in the regulation of asexual and sexual development, the *veA1* strain produces a somewhat less amount of LaeA-regulated secondary metabolites, such as sterigmatocystin (Kim et al. 2002). Deletion of *veA* gene results in the complete lack of sexual development (neither Hülle cells nor cleistothecia are produced) and lack of sterigmatocystin production.

During the practical course all the *A. nidulans* strains we work with carry the *veA1* allele.

Terminology: auxotroph and prototroph strains

The prototroph strains are able to grow on minimal medium, which contains only salts, trace elements, a simple N-source (e.g. nitrate, ammonium, acetamide, hypoxanthine, xanthine, urea, uric acid etc.) and a simple C-source (*e.g.* glucose, lactose, galactose *etc.*). The auxotroph strains carry mutation in gene(s), which is/are implicated in the biosynthesis of a particular vitamin, amino acid, nucleobase or any other essential organis compound. The auxotroph strains cannot grow on a minimal medium unless the particular vitamin, amino acid, nucleobase or the particular non-synthesized compound is added to the medium. Auxotroph mutants can be obtained by spontaneous mutation(s) or induced mutagenesis.

Genetic cross between two genotypes in practice

In order to cross two strains with different genotypes, the chosen strains must be auxotroph, and the auxotrophy must be different. Thus the parental strains can complement each other's auxotrophy in a heterkokaryon. This also means that none of the parental strains can grow on minimal medium itself. However, heterokaryons, arisen from anastomosis between the two parental hyhae (on supplemented medium where both parent are able to grow), can grow when being transferred onto minimal medium and form heterokaryotic colonies. Anastomosis occurs regularly between two closely positioning hyphae and upon selection pressure (lack of additional supplements in the medium, such as vitamins) they are forced to keep the heterokaryotic form.

Although different auxotrophy of the parents are pivotal for the genetic cross, using partners with different conidiospore colors are not necessary, but useful. It makes the identification of heterozygotic cleistothecia easy and additionally, measuring the ratio of the differently colored conidiospores makes possible to evaluate the balance of the parental nuclei in the heterokaryon. Remember, that a conidiospore derives by the budding of a uninucleate phialide. Therefore the nucleus of a conidiospore comes either from one or the other parent. It means that colonies, which are formed by the germination and growth of a single conidiospore derived from a heterokaryon must be always homokaryotic. Thus heterokaryon colonies cannot be maintained through the inoculation of conidiospores. The only way to transfer a heterokaryon colony from one plate of minimal medium to another is to take sample from the heterokaryotic mycelia and transfer it to the new, minimal medium plate. Conidiospores taken and transferred accidentally to the new plate will not germinate on the minimal medium, except in case when the conidiospore carry a heterozygous diploid nucleus.

The dikaryotic ascogenous hyphae inside the maturing fruiting body can be homozygous or heterozygous. In case the dikaryotic ascogenous hyphae in the fruiting body are homozygous, the meiotic events occour between genetically identical nuclei, therefore the result of the meiotic events will be the generation of parental ascospores. In case the dikaryotic ascogenous hyphae in the fruiting body are heterozygous, the meiotic events occour between genetically different nuclei, therefore the result of the meiotic events will be the generation of recombinant ascospores. Please note, that generally the cleistothecia with uniparental origin are always very small in size, while the recombinant cleistothecia are sometimes ten times bigger. To determine the parental or recombinant nature of collected cleistothecia, we test the growth ability of the ascospores on non-supplemented minimal medium, where only prototroph progeny derived from recombination events between the two parental nuclei can grow.

In practice, the first step of a crossing is the inoculation of the parental strains on the same plate with complet media in close proximity to each other. We grow them up until the perimeter of the growing parental colonies will physically contact (Figure 8, panel A).

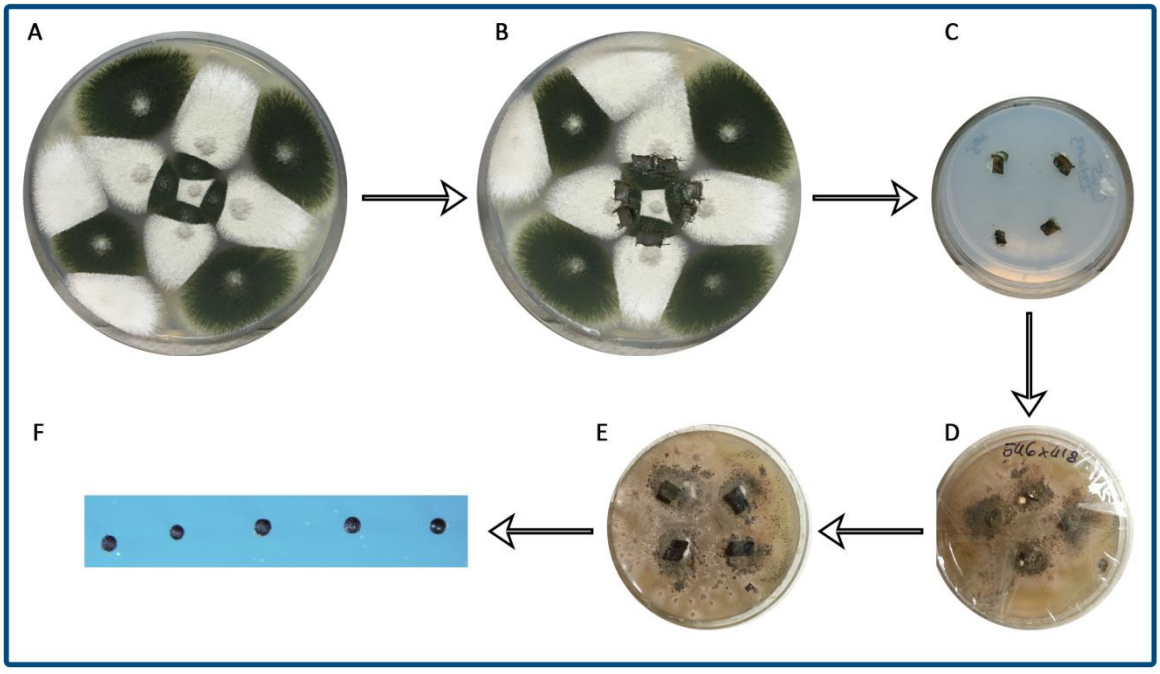

Figure 8: Execution of genetic cross between *A. nidulans* strains carrying different auxotrophies.
A: Parental strains are inoculated on complete medium side-by-side in close proximity to each other.
B: Agar blocks carrying both colonies are cut from the plate. Samples are taken from those regions where the two colonies are grown together tightly. C: The cut agar blocks are placed face-down onto the surface of a non-supplemented minimal medium. After 2 days of incubation the plate is tightly sealed with cellux tape.
D: After two weeks of incubation the grown up heterokariotic colony is covered with cleistothecia.
E: After opening the plate cleistothecia are collected and purified by rolling them on the surface of an empty sterile plate with minimal medium. F: The cleaned cleistothecia are free from mycelia, conidiospores or Hülle cells. They are shiny black balls with regular rounded shape. Pictures were done by Eszter Bokor.

The second step is cutting out agar blocks from the area where the two colonies connect each other (Figure 8, panel B). The agar block must be placed face down on the surface of minimal medium, where none of the parental strains can grow (Figure 8, panel C). After 2-3 days of incubation the heterokaryon will appear and grow. As a third step we exclude the air change by tightly sealing the plate with cellux tape. The heterokaryon grows further and upon increase of the CO_2 level the sexual development is initiated. Since we are working with *veA1* mutant, we do not need to keep the plates in dark in order to initiate the sexual development. After 1 week, white globosus nests will be produced, which mature to µ-cleistothecia and cleistothecia for the end of the second week of incubation. After the second week we open the plates and collect the large dark-purple/blackish cleistothecia (Figure 8, panel D-F). The cleistothecia must be purified by rolling them on the surface of agar medium until they are shiny black and free from conidiospores, Hülle cells and mycelial contaminations (Figure 8, panel F). The purified cleistothecia must be placed into eppendorf tubes containing destilled water or 0.01% Tween-80 solution. The cleistothecia are opened by pressing them to the wall of the tube with the tip of a pipette. When the ascospores are released from the broken fruiting body, the liquid gains purple color. Small portion of the ascospores must be tested for growth by striking them on minimal medium in order to determine the parental or recombinant nature of the corresponding cleistothecium. After 2 days of incubation, those samples, which show growth, are derived from recombinant cleistothecia. Progeny analysis must be executed on ascospores, which were derived from recombinant cleistothecium.

How to write and read genotypes? – The nomenclature of A. nidulans

Gene names are written with italic letters and first letter must be with small case (e.g. *riboB*). Proteins are written with normal style letters, the first letter is always capital (RiboB). In case genes belong to the same gene-family or the same biological process, a capital letter of the alphabet is added at the end of the gene family name. For example the genes involved in the biosynthesis of the vitamin riboflavine are called *riboA*, *riboB, riboC, riboD, riboE, riboF, riboG* and *riboH.* These gene-names correspond to the wild type alleles. In case there is a mutation in the gene, an arabic number is written after the wild type formula (e.g. *riboB2*). When a gene is deleted from the genome, the greek delta letter is written after the wild type formula (*riboBΔ*).
The nomenclature is always uniform for a species but might be different between different species!

Color of the conidiospores

Conidiospores of wild type *A. nidulans* are green. The compound, which colorizes the conidiospores protects the spores from the harmful UV radiation. Two main genes are responsible for the biosynthesis of the green colored compound. They are the *wA* and *yA* genes. The *wA* gene codes for a polyketide synthase, which is responsible for the production of the yellow precursor compound. The *yA* codes for a laccase (p-difenol oxydase) expressed in conidia, which transforms the yellow colored precursor compound to a green product. Mutation in *yA* gene (e.g. *yA2*) causes yellow coloured conidiospores, mutation in *wA* (e.g. *wA3*) results in white coloured conidiospores. A double mutant strain *yA2 wA3* carries white conidiospores, which indicates the order of the colour formation, which occures in two steps: white $\rightarrow$ yellow $\rightarrow$ green.

Vitamin auxotrophy used during the course

We work with pantothenic-acid (panto) and para-aminonenzoic acid (paba) auxotroph mutants during the practical course. Alleles responsible for these auxotrophies are *pantoB100* and *pabaA1*. The *pantoB100* mutant can grow only on minimal medium if it is supplemented with the panto vitamin. In case of the *pabaA1* mutant, the minimal medium must be supplemented with paba in order to grow.

Literature

Bayram Ö, Krappmann S, Ni M et al. (2008) VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320: 1504–1506.

Bayram Ö & Braus GH (2012) Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. FEMS Microbiol Rev. 2012 36(1):1-24

Braus GH, Krappman S & Eckert SE (2002) Sexual development in ascomycetes. Fruit body formation of *Aspergillus nidulans*. Molecular Biology of Fungal Development (Osiewacz HD, ed), pp. 215–244. Marcel Dekker, New York.

Busch S, Schwier EU, Nahlik K, Bayram O, Helmstaedt K, Draht OW, Krappmann S, Valerius O, Lipscomb WN, Braus GH (2007) An eight-subunit COP9 signalosome with an intact JAMM motif is required for fungal fruit body formation. PNAS 104(19):8089-94.

Carvalho MDF, Baracho MS & Baracho IR (2002) Investigation of the nuclei of Hülle cells of *Aspergillus nidulans*. Genet Mol Biol 25: 485–488.

Graafmans WDJ (1973) The influence of carbon dioxide on morphogenesis in *Penicillium isariiforme*. Archiv fur Mikrobiologie 91: 67-76.

Long PE & Jacobs L (1974) Aseptic fruiting of the cultivated mushroom, *Agaricus bisporus*. Transactions of the British Mycological Society 63: 99-107.

Han KH (2009) Molecular genetics of *Emericella nidulans* sexual development. Microbiology 37: 171–182.

Han KH, Lee DB, Kim JH, Kim MS, Han KY, Kim WS, Park YS, Kim HB & Han DM (2003) Environmental factors affecting development of *Aspergillus nidulans*. J Microbiol 41: 34–40.

Käfer E (1965) Origins of translocations in *Aspergillus nidulans*. Genetics 52(1): 217-232.

Kim H, Han K, Kim K, Han D, Jahng K & Chae K (2002) The *veA* gene activates sexual development in *Aspergillus nidulans*. Fungal Genet Biol. 37(1): 72-80.

Ni M & Yu JH (2007) A novel regulator couples sporogenesis and trehalose biogenesis in *Aspergillus nidulans*. PLoS ONE 2: e970.

Park HS, Nam TY, Han KH, Kim SC, Yu JH (2014) VelC positively controls sexual development in *Aspergillus nidulans*. PLoS One 9(2):e89883. doi: 10.1371/journal.pone.0089883. eCollection 2014.

Pontecorvo G (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5: 141–238.

Rai JN, Tewari JP & Sinha AK (1967) Effect of environmental conditions on sclerotia and cleistothecia production in Aspergillus. Mycopathol Mycol Appl 31: 209–224

Sarikaya Bayram Ö, Bayram Ö, Valerius O, Park HS, Irniger S, Gerke J, Ni M, Han K-H, Yu J-H & Braus GH (2010) LaeA control of velvet family regulatory proteins for lightdependent development and fungal cell-type specificity. PLoS Genet 6: e1001226.

Sietsma JH, Rast D & Wessels GH (1977) The effect of carbon dioxide on fruiting and on the degradation of a cell-wall glucan in *Schizophyllum commune*. Journal of General Microbiology 102: 385-389.

Sohn KT & Yoon KS (2002) Ultrastructural study on the cleistothecium development in *Aspergillus nidulans*. Mycobiology 30: 117–127.

Stinnett SM, Espero EA, Cobeno L, Araujo-Bazan L & Calvo AM (2007) Aspergillus VeA subcellular localization is dependent on the importin a carrier and on light. Mol Microbiol 63: 242–255.

Yager LN (1992) Early developmental events during asexual and sexual sporulation in *Aspergillus nidulans*. Aspergillus: Biological and Industrial Applications (Bennett JW & Klich MA, eds), pp. 19–42. Butterworth, Boston, MA.

Wei H, Scherer KM, Singh A, Liese R & Fischer R (2001) *Aspergillus nidulans* α-1,3 glucanase (mutanase), mutA, is expressed during sexual development and mobilizes mutan. Fungal Genet Biol 34: 217–227.

Zonneveld BJM (1977) Biochemistry and ultrastructure of sexual development in Aspergillus. Genetics and Physiology of Aspergillus (Smith JE & Pateman JA, eds), pp. 59–80. Academic Press, London.

Zonneveld BJM (1988) Effect of carbon dioxide on fruiting in *Aspergillus nidulans*. Trans Br Mycol Soc 91: 625–629.

II. Overview of the course

1. Inoculation of the two parental strains HZS.420 and HZS.544 into an eppendorf tube containing complete medium.
2. Placing the floating mixed mycelial disk of the two parental strains onto the top of minimal medium.
3. Sealing the heterokaryon plates with minimal medium with cellux tape in order to cut off the oxygene.
4. Collecting of purified cleistothecia and testing the ascospores on minimal medium in order to find out whether they are parental or recombinant.
5. Streaking the ascospores of a recombinant cleistothecium onto the surface of complete media.
6. Manual replica plating of colonies from the complete media onto minimal media supplemented with vitamins in various combinations.
7. Full progeny analysis: determining the genotypes of the analysed progeny and judging whether there is linkage between the analysed alleles.

III. Practice 15 – from week 1 to week 7

1st week

Goal

Inoculation of the two parental strains HZS.420 and HZS.544 into an eppendorf tube containing complete medium.

Required materials

Used *A. nidulans* strains: HZS.420: *pantoB100 veA1*

HZS.544: *pabaA1 wA3 veA1*

3 days old culture of strains HZS.420 and HZS.544 incubated on complete medium; tooth ticks; 1.5 ml complete medium; components for 150 ml minimal medium (glucose, sodium-nitrate, 50x stock of *Aspergillus* salt solution supplemented with trace elements, agar, destilled water, infusion bottle, alufolie, high pressure table sterilizer, sterile cotton roll); 2 small and 3 large sterile plastic petri dish; 0.01% Tween80 solution; 6 pieces of sterile 1.5 ml eppendorf tubes with tiny aeration holes on their cap; eppendorf rack; container for the collection of hazardous cellular waste.

Tasks

Collecting conidiospores from strains HZS.420 and HZS.544 by pre-wetted tooth ticks and inoculation of the conidiospores into a single eppendorf tube containing 100 µl of 0.01% Tween80 solution. Try to collect equal number of conidiospores from each strains! Pipetting of 200 µl complete medium into 6 eppendorf tubes carrying an aeration hole on their cap and then pipetting 1 µl conidiospore suspensions of the parental strains into the tubes. Incubation of the cultures for 2 days at 37°C.

Preparation of 150 ml minimal medium (1% glucose, 10 mM $NaNO_3$, 1 x salt solution, 2.5% agar) and pouring 2 small and 3 large petri dishes from the autoclaved minimal media. The conidiospore suspensions must be stored at 4°C until manual replica plating.

Media in the small petri dishes must be tall, leave only 2 mm distance from the top edge of the plate! In case of the large plates, half-filled petri dishes are required. The petri dishes with the media must be kept on 4°C until usage.

After 2 days of incubation the mycelium disks composed from the mixed hyphae of the parental strains must be transferred onto the top of the small petri dishes with minimal media by a sterile tooth tick. Three disks must be placed onto the surface of each small minimal medium by equal distance from each other.

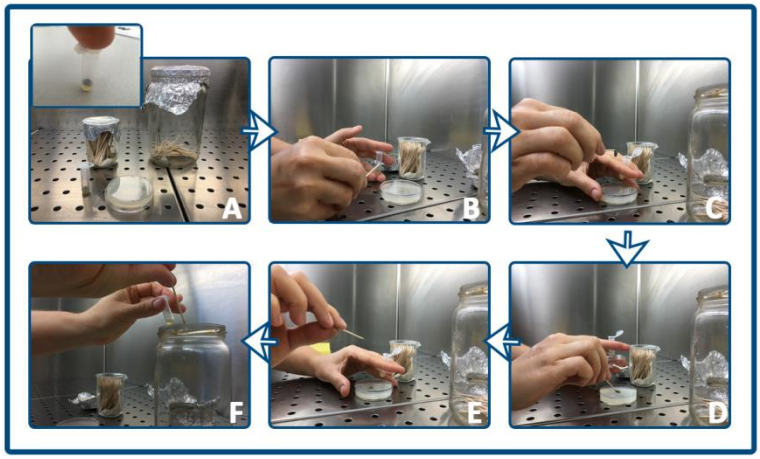

Figure 9: Transfer of mycelium disks composed from the mixed hyphae of the parental strains onto the top of the small petri dish with minimal media by using a sterile tooth tick.

2nd week

Goal

Monitoring of the heterokaryon formation on the minimal medium and exclusion of the air by sealing of the plates.

Required materials

Stereo microscope; cellux tape; scissors.

Tasks

Observation of the growing heterokaryons under stereo microscope. Checking the presence of white and green colored conidiospores and estimation of their balanced or unbalanced distribution in the heterokaryon. Double-sealing of the petri dishes by cellux tape. Incubation of the heterokaryons for 2 weeks at 37°C.

3rd week

Goal

Monitoring of the 1 week old heterokaryons.

Required materials

Stereo microscope.

Tasks

Monitoring of the 1 week old heterokaryons under stereo microscope. The heterokaryons keep on growing. When the oxygene tension drops, the sexual development is induced, Hülle cells are produced, the primordiums are formed and finally the cleistothecia. Observation of nest formation and unmature cleistothecia under stereo microscope. Mature cleistothecia appear as black/dark purple balls at the end of the second week (Figure 8, panel F).

4th week

Goal

Collecting of purified cleistothecia and testing the ascospores on minimal medium in order to find out whether they are parental or recombinant.

Required materials

Stereo microscope; large petri dishes with minimal media maden at the first practice; sterile tooth ticks; 0.01%-os Tween80 solution; container for the collection of hazardous cellular waste; 1-20 µl pipette; 20-200 µl pipette; sterile pipette tips; eppendorf rack; sterile eppendorf tubes.

Tasks

Opening of the sealed heterokaryon plates and collecting 4 large cleistothecia by sterile tooth tick and placing them on the surface of a sterile agar plate with minimal medium. Purification of each cleistothecium by rolling them on the surface of the agar plate by a sterile tooth tick under stereo microscope. The cleistothecia must bee free from Hülle cell, conidiospore and mycelial contamination. They must be looked as shiny dark balls. The purified cleistothecia must be separately transferred into eppendorf tubes containing 100 µl 0.01%-os Tween-80 solution. After that the cleistothecia must be ruptured by pressing them to the wall of the tube with a sterile tip of the 20-200 µl pipette. Upon rupture, the purple ascospores are released. Try to eliminate the remnants of the wall of the cleistothecia by the tip of the pipette. The ascospore suspensions must be store at 4°C until usage.

The cleistothecia must be tested whether they are parental or recombinant. In the test 1 large Petri dish with minimal medium is used. Mark 4 quarter areas at the bottom of the plate by marker pen and inoculate 5 µl ascospores from the suspensions separately into the quarter areas by streaking the samples with a sterile pipette tip. After that the plates must be kept on the bench for 10-15 minutes before placing them in upside-down position into the 37°C incubator for 3 days. In case a cleistothecium is parental, no ascospores can grow on the minimal medium. In case the cleistothecium is recombinant, the genetic markers are recombined in the progeny, therefore among the progeny of parents carrying different vitamin auxotrophies, prototrophs will be generated. Thus prototroph colonies will grow on the minimal medium. Since the colour of the

conidiospores of the parent were green and white, the two different colour must occour in the growing prototroph progeny on the test plate. The appearance of the 2 colours in the progeny also confirms the recombinant origin of the fruit body and its ascospores. One of the recombinant cleistothecia must be chosed for the further experiments.

5th week

Goal

Streaking the ascospores of a recombinant cleistothecium onto the surface of complete media.

Required materials

Recombinant ascospore suspension; 100 ml complete medium; 100x vitamin solution to supplement the complete medium; large Petri dishes; 0.01% Tween80 solution; ; container for the collection of hazardous cellular waste; 1-20 µl pipette; 20-200 µl pipette; 100-1000 µl pipette; sterile pipette tips; eppendorf rack; sterile eppendorf tubes, glass streaker; dish with alcohol.

Tasks

After autoclave sterilization of the solid complete medium 3 large Petri dishes must be made. One of the recombinant ascospore solution must be diluted by 500 times fold (add 1 µl ascospore suspension to 499 µl 0.01%-os Tween80 solution) and 30-, 50-, and 70 µl ascospore solutions must be streaked to the surface of the complete media by using a glass streaker. The plates must be incubated in upside-down position at 37°C for 3 days.

6th week

Goal

Manual replica plating of colonies from the complete media onto minimal media supplemented with vitamins in various combinations.

Required materials

Conidiospore suspension of the parental strains HZS.420 and HZS.544 made during practice 1 and stored at 4 °C; progeny on complete media from last week practice; container for the collection of hazardous cellular waste; sterile tooth ticks; sterile eppendorf tubes; eppendorf rack; 0.01%-os Tween80 solution; components for minimal medium (glucose, sodium-nitrate, 50 x stock of *Aspergillus* salt solution supplemented with trace elements, agar, destilled water, infusion bottle, alufolie, high pressure table sterilizer, sterile cotton roll); 100 x stock of p-amino benzoicacid (paba) and Ca-D-pantothenate (panto); template for manual replica palting

Tasks

1 large Petri dish with minimal media (MM) supplemented with the relevant vitamins in different combinations must be made (MM without panto and paba, MM supplemented with 1 x paba and 1 x panto, MM supplemented with 1 x paba, MM supplemented with 1 x panto). Before usage, dry the surface of the medium under sterile cabinet in order to achive droplet-free surface. Use the template for the replica plating to draw the patter onto the bottom of the plates by a marker pen. Prepare 17 eppendorf tubes with 30 µl 0.01% Tween-80 solution and collect conidiospores from 17 distinct colonies (50% white and 50% green) by using pre-wetted sterile tooth ticks. Use the conidiospore suspensions of the 2 parental strains and the 17 progeny to inoculate the 4 different medium in the same pattern by using sterile tooth ticks. For inoculation the hand holding the tooth tick with the inocula must be palced in fixed position on the bench, the tooth tick facing upward. The plate must be in upside down position on the bench, and the half part of the dish carrying the medium must be brought above the position where the inoculation must be done. The plate must be moved downward until the toothtick touches the surface of the medium and then the half dish must be replaced into its top part, upside-down. Inoculation of the 4 plates must be made in the same pattern! The plates must be incubated in upside-down position at 37 °C. After 3 days of incubation the plates must be stored at 4 °C until usage.

7th week

Goal

Full progeny analysis: determining the genotypes of the analysed progeny and judging whether there is linkage between the analysed alleles.

Required materials

Replica plates from the last week.

Tasks

Reading out the plates by using the Table below. Mark growing with + and lack of growth with - marks. Give the full genotype of each progeny and make a comment whether you found linkage between genetic alleles or not.

Cross: HZS.420 X HZS.544						
Media / Strains	MM	MM paba	MM panto	MM paba panto	color of conidia	GENOTYPE
HZS.420						
HZS.544						
progeny 1						
progeny 2						
progeny 3						
progeny 4						
progeny 5						
progeny 6						
progeny 7						
progeny 8						
progeny 9						
progeny 10						
progeny 11						
progeny 12						
progeny 13						
progeny 14						
progeny 15						
progeny 16						
progeny 17						

Do you think linkage between any of the alleles?

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.....

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.....

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Check your progress

After the completion of the practical course, answer the following questions.

- **What does it mean:**
  - **homothallic,**
  - **heterothallic**
  - **dikaryon**
  - **heterozygous dikaryon**
  - **homozygous dikaryon**
  - **homokaryon**
  - **heterokaryon**
  - **anastomosis**
  - **karyogamy**
  - **heterozygotic cross**
  - **homozygotic cross?**
- **How would you define auxotrophy and how would you obtain auxotroph mutants?**
- **How can you determine the parental or recombinant nature of a cleistothecia?**
- **What is our purpose when we transfer mixed mycelia of two parental strains from complete medium onto non-supplemented minimal medium?**
- **How do we transfer a heterokaryotic mycelium from one plate to the other? What happens with transferred conidiospores and why?**
- **By streaking conidiospores collected from heterokaryon of a green and white strain, which color will you find on the germinated colonies?**

  A. only green

  B. only white

  C. green colonies and white colonies

  D. mixed green and white colonies
- **What can be genotype of a white progeny derived from the genetic cross of *yA2* x *wA3*?**

3.1.3.4. The role of light in the coordination of asexual development, sexual development and secondary metabolite production of *A. nidulans* – Practice 16 -

I. Introduction

- ***Light dependent development of A. nidulans***
- ***Role of VeA protein in the light dependent regulation of asexual development, sexual development and secondary metabolite production***
- ***Genetic background of sterigmatocystin production***

II. Overview of the course

III. Practice 16 – from week 1 to week 2

- **1st week: Inoculation of *veA1* and *veA+* strains for phenotype study and sterigmatocystin extraction**
- **2nd week: Study of the conidia and cleistothecia formation of *veA1* and *veA+* strains incubated in dark and light and comparison of the sterigmatocystin content of these differently cultured strains**

Learning Objectives

After the completion of the practical course, you should be able to:

- **Explain the role of light in the regulation of asexual and sexual reproduction of *A. nidulans***
- **The role of VeA protein in the light dependent coordination of development**
- **The impact of *veA1* mutation on the regulation of light dependent development and secondary metabolite production of *A. nidulans***
- **Explain the linkage between sexual development and sterigmatocystin production**
- **Define the following terms: nuclear localisation signal, gene cluster, thin layer chromatography**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, learning sterilization techniques, usage of automatic pipettes, usage of sterile cabinets, preparation of culture media, usage of stereomicroscope, inoculation of filamentous fungi, culturing of filamentous fungi on solid media, extract of secondary metabolites; thin layer chromatography**

I. Introduction

Light dependent development of A. nidulans

Light is a key environmental factor that is sensed by the fungus via phototreceptors. *A. nidulans* possesses red-light sensing phytochrome (FphA), blue-light signaling white collar-like proteins (LreA and LreB) and UV-A cryptochrome (CryA) (Bayram *et al*., 2010). FphA and CryA repress sexual reproduction under red and UV-A or blue light wavelengths, respectively, while LreA and LreB activate sexual reproduction in the dark and also promote limited sex in the light. Red- and blue-light receptors are assembled into a light-sensing protein complex and the signal from the sensory complex is transmitted to various regulatory proteins, such as the velvet protein VeA, the key regulator of (asexual- and sexual) development and secondary metabolism. Light stimulates asexual development and conidiogenesis in a wild-type strain, while the sexual development is suppressed. In dark, however the sexual development is initiated, while the asexual reproduction is suppressed.

Role of VeA protein in the light dependent regulation of asexual development, sexual development and secondary metabolite production

In the light the VeA protein resides in the cytoplasm. In dark, the VeA protein in complex with another velvet protein, VelB, enters the nucleus through the KapA importin and forms a heterotrimeric complex with VelB and LaeA inside the nucleus (Figure 1). This complex named as ’velvet complex’ activates both sexual development and sterigmatocystin (secondary metabolite) production (Figure 1). VelB also interacts with VosA (another velvet protein) and the heterodimer contributes to spore viability and also represses asexual development (Figure 1). LaeA activated by VeA regulates many clusters of secondary metabolite biosynthesis, including the cluster of sterigmatocystin biosynthesis. Thus, the VeA protein couples two biolgical processes, sexual development and secondary metabolite production through bridging the VelB and LaeA in the ’velvet complex’.

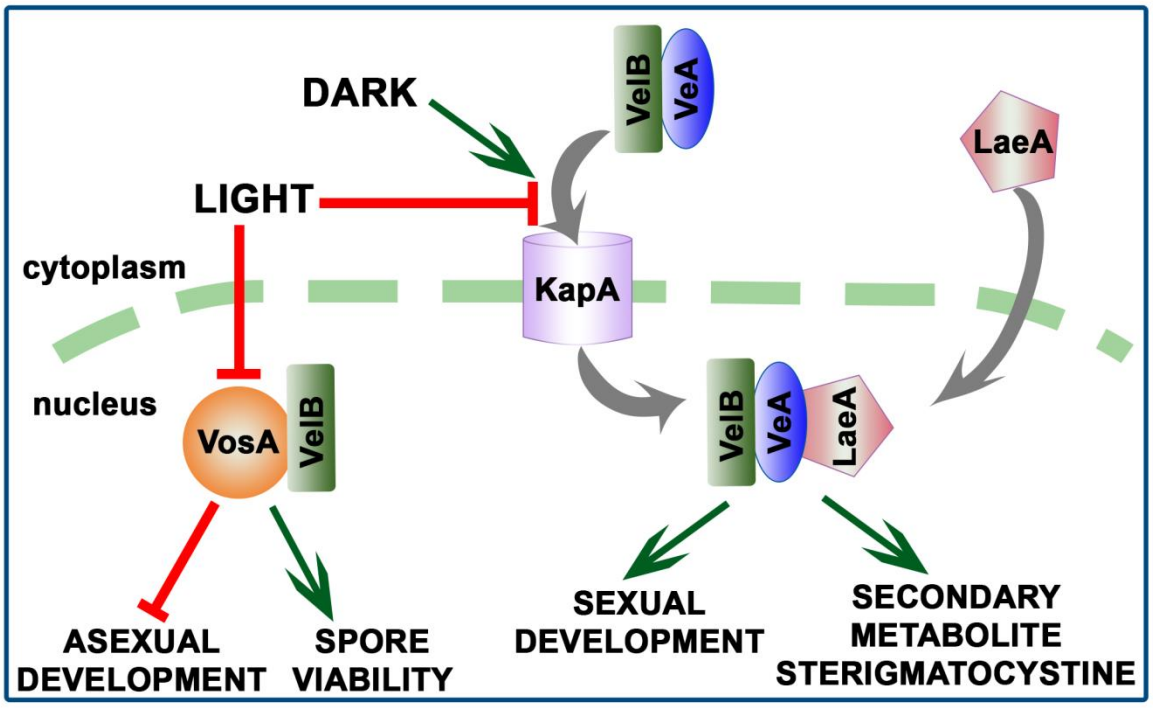

Figure 1: Light dependent assembly of complexes of velvet family regulatory proteins VeA, VelB and VosA. VeA primarily enters the nucleus together with VelB through KapA α-importin. Then, VelB can be distributed to two distinct complexes. The VosA-VelB dimer represses asexual development (conidiogenesis) and facilitates spore maturation (e.g. by activating trehalose biogenesis). VeA-VelB can associate with LaeA and the trimeric complex (called velvet complex) controls sexual development and secondary metabolite production.

In a *veA* deleted strain the sexual development is impaired, Hülle cells, ascogenous hyphae and cleistothecia are not formed, and the strain does not produce sterigmatocystin (Kim et al., 2002). In the *veA1* mutant the first 36 amino acids of the protein carrying the nuclear localisation signal are missing and the truncated protein fails to be imported by the KapA importin. The truncated protein in *veA1* strains shows a predominant cytoplasmic location, which is not altered by illumination. The *veA1* strains produce conidiospores abundantly even in the dark, form fewer cleistothecia compared to wild-type by an illumination-independent manner and produce nearly as much sterigmatocystin as the wild-type strain.

Genetic background of sterigmatocystin production

Genes of sterigmatocystin biosynthesis pathway form a 60 kb gene cluster (*stc* cluster) on chromosome IV that includes 25 co-regulated genes (Figure 2) (Brown *et al.*, 1996). The *aflR* gene is a pathway-specific transcription factor, which acts as the cluster-specific regulator for the activation of the other genes in the *stc* cluster.

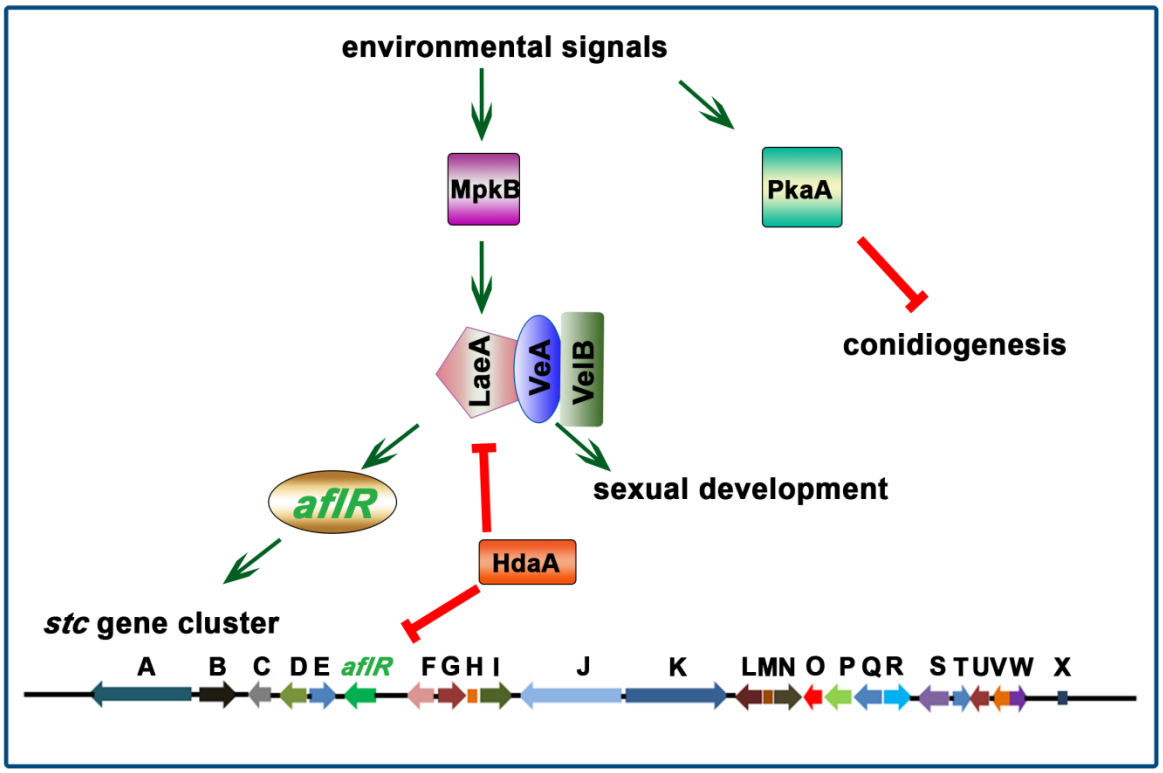

Figure 2: Regulation of sterigmatocystin production in *A. nidulans* (adopted from Fox and Howlett, 2008). The schematic figure explains the regulation process of sterigmatocystin production through activation of many regulators such as MpkB, velvet family proteins (VelB, VeA), LaeA and master regulator AflR. Histone deacetylase HdaA represses LaeA functions and inghibits sterigmatocystin production. PkaA: Protein kinase A, a member of the PkaA signal transduction pathway that upon activation inhibits asexual sporulation. Arrows represent gene activation, while blocked arrows represent repression. At the bottom the schematic representation of the sterigmatocystin biosynthesis *stc* gene cluster is shown.

The expression of *aflR* is regulated during the early stationary phase of the life cycle and depends on LaeA and VeA functions (Yu *et al.*, 1996). *AflR* expression and thus the sterigmatocystin biosynthesis is regulated at chromatin level by the methyltransferase LaeA (Figure 2) (Bok and Keller, 2004). LaeA, is a global regulator of secondary metabolism, which contains a classical NLS region located at the N-terminus of the protein that results in its nuclear localization. On the other hand, LaeA is part of the velvet complex that control development and thereby LaeA plays role in coupling developmental processes and secondary metabolite production (Sarikaya Bayram *et al*., 2010). The *A. nidulans* putative mitogen-activated protein kinase, encoded by *mpkB,* is necessary for normal expression of *laeA* to regulate cluster genes for sterigmatocystin production (Atoui *et al*., 2008) (Figure 2).

Literature:

Atoui A, Bao D, Kaur N, Grayburn WS. and Calvo AM (2008) *Aspergillus nidulans* natural product biosynthesis is regulated by *mpkB*, a putative pheromone response mitogen-activated protein kinase. Applied and Environmental Microbiology, 74(11): 3596–3600.

Bayram O, Braus GH, Fischer R, Rodriguez-Romero J (2010) Spotlight on Aspergillus nidulans photosensory systems. Fungal Genet. Biol. 47(11): 900–908.

Bok JW and Keller NP (2004). LaeA, a regulator of secondary metabolism in *Aspergillus spp*. Eukaryot. Cell. 3: 527–535.

Brown DW, Yu JH, Kelkar HS, Fernandes M, Nesbitt TC, Keller NP, Adams TH, Leonard TJ (1996) Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. Proc Natl Acad Sci U S A. 93(4): 1418-1422.

Fox EM and Howlett BJ (2008). Secondary metabolism: regulation and role in fungal biology. Current Opinion in Microbiology, 11(6): 481–487.

Kim H, Han K, Kim K, Han D, Jahng K & Chae K (2002) The *veA* gene activates sexual development in *Aspergillus nidulans*. Fungal Genet Biol. 37(1): 72-80.

Sarikaya Bayram O, Bayram O, Valerius O, Park HS, Irniger S, Gerke J, Ni M, Han KH, Yu JH, Braus GH (2010) LaeA control of velvet family regulatory proteins for light-dependent development and fungal cell-type specificity. PLoS Genet. 6(12):e1001226.

Yu J H, Butchko R A, Fernandes M, Keller N P, Leonard T J, Adams T H (1996). Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. Curr Genet. May; 29(6):549–555.

II. Overview of the course

1. Inoculation of *A. nidulans* strain HZS.450 (*veA*$^{+}$) and HZS.145 (*veA1*)

2. Comparison of growth properties of the *veA*$^{+}$ and *veA1* strains. Extraction of sterigmatocystin and visualisation of sterigmatocystine content in the samples by using thin layer chromatograph.

III. Practice 16 – from week 1 to week 2

1st week

Goal

Inoculation of a *veA*$^{+}$ and a *veA1* strain for the purpose of detection of sterigmatocystin production.

Required materials

Used *A. nidulans* strains: *veA1* mutant HZS.145: *veA1*; *veA*$^{+}$ control HZS.450: *riboB2.*

Compounds for the preparation of 100 ml minimal medium with nitrate nitrogen-source and glucose carbon-source (glucose, sodium nitrate, 50× salt solution stock, 100× vitamin solution stock, agar); sterile Petri dish (4 pieces); 3 days old cultures of HZS.145 and HZS.450; sterile tooth ticks; glass container; pipette set; sterile tips for pipettes; sterile eppendorf tubes; sterile 0.01% Tween-80 solution; alufolie, sterile 15 ml Falcon tubes (4 pieces); 1 piece of 10 cm long glass test tube with aluminium cap.

Tasks

Prepare 100 ml liquid minimal medium with nitrate nitrogen-source (5 mM) and glucose carbon-source (1 %) supplemented with vitamins (1×). Separate 90 ml medium in an infusion glass bottle supplemented with agar (2.5%) and 10 ml medium in a without agar. After sterilization by using high pressure cooker, supplement the media with vitamins and prepare 4 plates from the solid medium and place 1 ml medium into the 15 ml Falcon tubes. Prepare conidiospore solution from HZS.145 and HZS.450 and inoculate each strain to 2 plates and 2 Falcon tubes. Cover 1 plate and 1 Falcon tube with alufolie and leave the other plate and Falcon tubes uncovered. Incubate one set of cultures in dark and one set of cultures in light for 6 days at 37 °C .

2nd week

Goal

Extraction of sterigmatocystine and comparative analysis of produced sterigmatocystine in *veA*$^{+}$ and a *veA1* strains grown under different light conditions by using thin layer chromatography (TLC).

Required materials

10 ml chloroform; Kieselgel 60 TLC silica gel; pipette set; tips for pipettes; 1 % $AlCl_3$ dissolved in methanol; 40 ml 4:4:1 TEF (toluol:ethylacetate:formic acid) solution; glass chamber for developing the TLC plates; hair dryer machine, 15x15 cm sized glass tray; UV lamp; pincers; glass beads (with 0.2-0.5 mm diameter)

Tasks

1. Compare the conidiospore productivity of HZS.145 and HZS.450 incubated in dark and light! Describe your observations in a few sentences and discuss your result with those results that were expected on the basis of literature.

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2. Place 1 ml chloroform in each 15 ml Falcon tube containing the 6 days old cultures and add a few glass beads to the samples. Vortex the samples for 5 minutes. Collect the organic phase into an eppendorf tube by using a pipette. Place 4×5 µl sterigmatocystin containing chloroform drops of the samples side by side and add a drop from sterigmatocystin standard. Develop the chromatogram in 5:4:1 TEF solution in a glass chamber with covered top. When the solvent reaches the top part of the silica gel plate, take out the plate and let it dry under a hood. Pour out the 1% $AlCl_3$ solution into a glass tray and submerge the plate in it for a moment. Take out the plate and dry it immediately by using a hair dryer machine. Place the silica gel plate under UV light and document the chromatogram.

Please, answer the following questions!

Which kind of color is characteristics for the sterigmatocystin compound developed according to the above executed protocol?

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Do you detect difference in the sterigmatocystine content of HZS.450 and HZS.145 incubated in dark and light?

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Discuss your results with those results that were expected on the basis of literature!

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Check your progress

After the completion of the practical course, answer the following questions.

- **What does it mean:**
  - secondary metabolite
  - gene cluster
  - nuclear localization signal
- **What is the role of the velvet complex in *A. nidulans*?**
- **List the components of the velvet complex!**
- **How do you execute sterigmatocystin extraction?**
- **How do you develop a Thin Layer Chromatogram?**
- **How do you visualize the presence of sterigmatocystin on a Thin Layer Chromatogram?**

3.1.3.5. Parasexual life cycle of *A. nidulans* -Practice 17-

I. Introduction

- ***Diploid formation***
- ***Life cycle of dikaryotic mycelia; haploidization through the formation of aneuploids***

II. Overview of the course

II. Practice 17 – from week 1 to week 3

- **1st week: Formation of heterokaryons by protoplast phusion**
- **2nd week: Isolation of diploids**
- **3rd week: Haploidization**
- **4th week: Isolation and genetic analysis of haploid sectors segregated from the diploid colonies**

Learning Objectives

After the completion of the practical course, you should be able to:

- **Understand and explain the haploidization process through the formation of aneuploids due to series of chromosome non-disjunctions**
- **Define the following terms: heterokaryon, heterozygous dikaryon, heterozygous diploid, haploidization, aneuploid, chromosome non-disjunction**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, learning sterilization techniques, usage of automatic pipettes, usage of sterile cabinets, preparation of culture media, inoculation of filamentous fungi, culturing of filamentous fungi on solid media, portoplast formation from the filamentous fungus *Aspergillus nidulans*, selection for diploids**

I. Introduction

Diploid formation in A. nidulans

In a heterokaryotic mycelium karyogamy between two parental nuclei may happen with a very low frequency that will result in a diploid nucleus (Figure 1). When a diploid nucleus participates in conidiospore genesis, diploid conidiospores will be generated (Figure 1). Formation of diploid nucleus is a rare event, with a 10^{-5}-10^{-8} frequency. It means that one diploid conidiospore can be found in every 10^{5}-10^{8} conidiospores. Identification of diploid nuclei is rather easy if we create heterokaryon of two strains, one of them with *wA3* mutation (result in white conidiospores) and the other with *yA2* mutation (result in yellow conidiospores). The heterokaryon of the white and yellow strains will result in the formation of white and yellow haploid conidiospores. However, in case a *wA3* and *yA2* nuclei fuse together and form a diploid nucleus, the color mutations will complement each other in the diploid. If this diploid nucleus participates in the sterigmata formation (metula and phialide), the conidiospores originated from a diploid phialide inherit the diploid nucleus (Figure 1). Color of the diploid nuclei of diploid conidiospores originated from *wA3* and *yA2* parental nuclei, will be green. Additionally, the diploid conidiospores have bigger size than those of with haploid nuclei. Similarly to the color mutations, the auxotroph mutations also complement each other in the diploid nucleus. Therefore collecting conidiospores from heterokaryotic colonies followed by the elimination of the accidentally collected heterokaryotic hyphae from the sample by filtration and spreading the collected and purified conidiospores on the surface of minimal medium that does not support the growth of the parental haploid conidiospores is a general method to isolate diploids.

Life cycle of dikaryotic mycelia; haploidization through the formation of aneuploids

The diploid conidiospores can germinate and form diploid mycelia. In the diploid hyphae the diploid nuclei are propagated by mitosis. During the mitosis of a diploid nucleus, occasionally the segregation of sister chromatids is not accurate and the sister chromatids fail to separate and segregate. This failure of separation and segregation of sister chromatids is called chromosome non-disjunction. As a result of chromosome non-disjunction, one progeny will carry 3 copies of

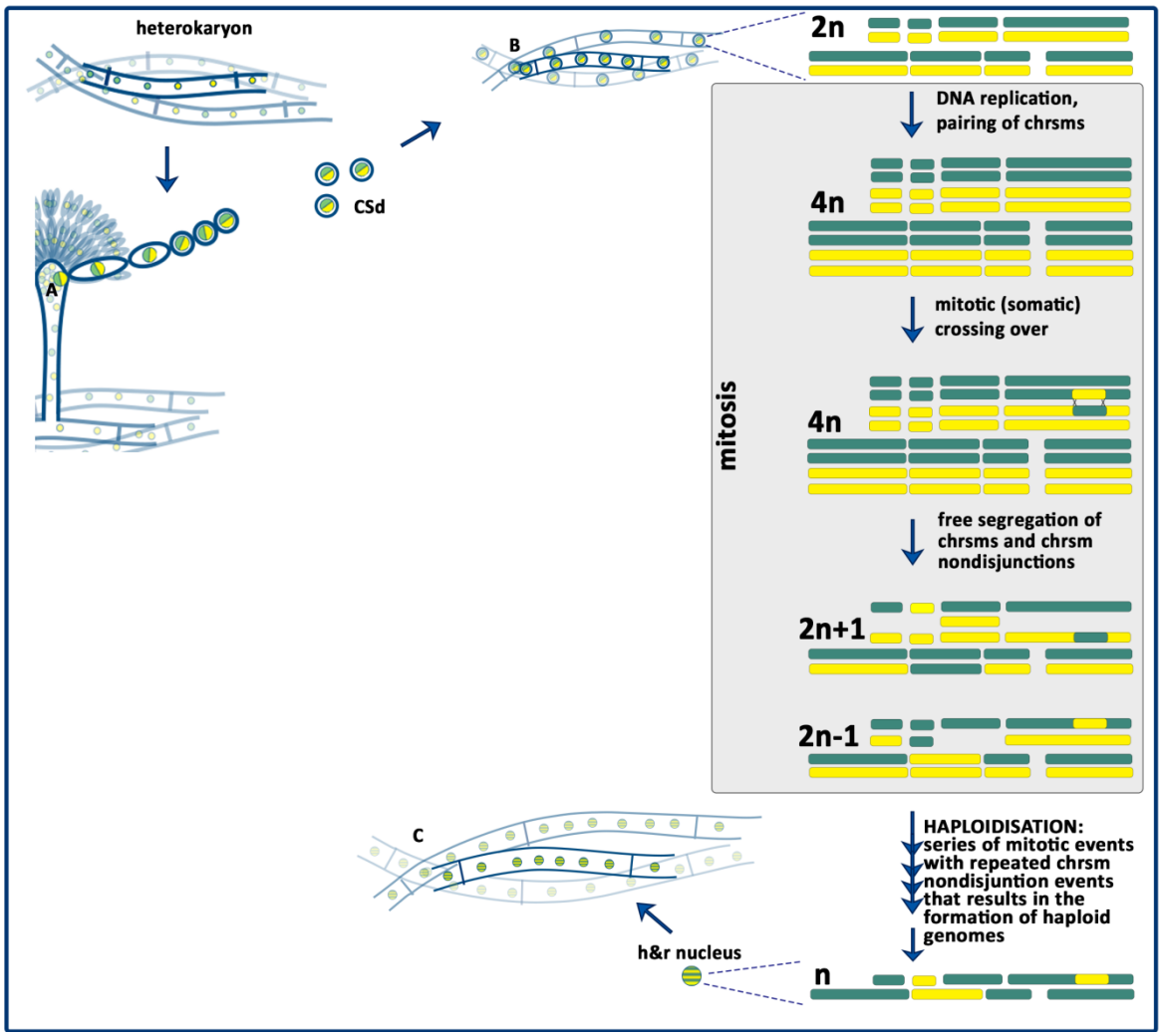


Figure 1: Parasexual life cycle of *A. nidulans*.
Rarely diploid nuclei are formed in the heterokaryon by the fusion of two haploid nuclei (A). When a heterozygous diploid nucleus plays role in metula cell formation, the metula cell and the subsequently formed phialide cell and all the conidiospores (CSd: diploid conidiospore) in the cognate spore chain will carry a single diploid nucleus. Germination of a diploid conidiospore will result in the formation of a diploid mycelium (B). When a heterozygous diploid nuclei undergo mitosis, the failure of the segregation of the sister chromatids, the so called chromosome non-disjunction will result in the loss of one or more of the chromosomes in the progeny. Grey boxed area shows an example of a mitotic event with mitotic crossing over between homologous chromosomes and chromosome non-disjunction that result in the formation of two progeny nuclei, one with 2n+1 and one with 2n-1 chromosome sets (aneuploids). Over several mitoses, the repeated events of chromosome non-disjunction will result in the haploidization of the diploid nuclei. The random losses of the parental chromosomes due to the chromosome non-disjunctions and the rarely occurring mitotic crossing overs will result in haploid genomes (n) with recombinant genetic material (h&r nucleus: haploid and recombinant nucleus). Participation of such h&r nucleus in conidiogenesis will result in the propagation of the h&r genome and will lead to the formation of new haploid, homokaryotic mycelia with genetically novel nuclear content (C).

the non-separated chromosomes and the other progeny will carry 1 copy of that chromosome (see example in Figure 1). The first progeny will have 2n+1 chromosome set (17 chromosomes), the other will have 2n-1 chromosome set (15 chromosomes). Ploidity of these progeny differs from both n (haploid) and 2n (diploids), therefore they are called aneuploids. If the nucleus of the second progeny (with 2n-1 chromosome set) will undergo a subsequent mitosis again with chromosome non-disjunction, then it will result in additional loss of chromosomes in one of the

two progeny, which will have 2n-2 chromosomes (aneuploid with 14 chromosomes). Mitosis after mitosis coupled with chromosome non-disjunction events will result in the regaining of the complete haploid chromosome set (8 chromosomes) in one of the descendants of the original diploid nucleus. Series of mitosis of a diploid nucleus that leads to haploid genome through the formation of aneuploids is called haploidization. Since the parental chromosomes are randomly lost in the aneuploids, the haploid genome is usually composed of the mixture of the parental chromosomes after haploidization (Figure 1). Technically the haploid progeny is recombinant.
In practice the haploidization of a diploid strain can be followed by having genetic markers on each chrormosomes. A sign of aneuploidia for example is the appearance of a white or yellow sectors in a green diploid colony originated from the heterokaryon of a *wA3* and a *yA2* parent.

Rarely, mitotic (somatic) crossing overs between non-sister homologous chromatids at the four-strand stage of mitosis occur with 10^{-3} frequency (Figure 1). Such events lead to the generation of progeny with recombinant genetic material. Study of mitotic crossing overs in diploids provided excellent tool for getting data about linkage of genetic markers before the era of robust, high throughtput genome sequencing methods.

In the laboratory, the failure of the segregation of the sister chromatides, the chromosome non-disjunction can be triggered by the usage of the inhibitor of the microtubules, benomyl.

Literature: Etta Kafer (1977) Meiotic and Mitotic Recombination in *Aspergillus* and Its Chromosomal Aberrations. In Advances in Genetics. Volume 19, Pages 33-131

II. Practice 17 – from week 1 to week 4

1st week

Goal

Obtaining protoplasts from the cellophane cultures of HZS.119 and HZS.544 and inducing fusion between protoplasts of the two parental strains followed by their inoculation to minimal media for isolation of heterokaryons.

Required materials

Used *A. nidulans* strains: HZS.119 (*yA2,anA1, riboB2, veA1*)
HZS.544 (*wA3, pabaA1, veA1*)

Required materials: Overnight (14 h) cellophane cultures of strains HZS.119 and HZS.544 prepared by the method described in section *3.1.2.2. Practice 9, 1st week*; forceps, 100 mg glucanex enzyme; 200 ml 0.7 M KCl; 2 sterile empty Petri dishes; 2 sheets of sterile cheese filters with 100 µm pore diameter; 2 sterile glasses with 500 ml capacity; 6 sterile cellophane capped glass centrifuge tubes with 25-30 ml capacity; 2 ml eppendorf tubes (at least 3 pieces); 0.2-1 ml pipette and tips; 0.02-0.2 ml pipette and tips; 12 aluminium capped glass test tubes with 4.5 ml 0.7 M KCl; TN1 solution (for 100 ml TN1: 5.22 g 0.7 M KCl, 0.735 g 50 mM $CaCl_2$); freshly prepared TN2 solution (for 5 ml TN2: 250 µl 1 M $CaCl_2$, 500 µl Tris/HCl, 3g PEG-4000); 6 Petri dishes with selective sucrose medium (SMM) (2% (v/v) salt solution; 1% (w/v) glucose, 10 mM $NaNO_3$, 1 M sucrose, 2.5% (w/v) agar, pH 6.8); 50 ml freshly prepared SMM top agar kept at 42 °C degree (SMM with 1% agar); water bath with 42 °C degree; one sterile 15 ml Falcon tube; 1 sterile 50 ml falcon tube or 20 cm long sterile glass test tubes with aluminium caps; microscope; haemocytometer (Burker chamber).

Tasks

Peel off the cellopane sheets with HZS.119 and HZS.544 cultures and place the three sheets into a single empty Petri dish. Soak the cellophane sheets in 5 ml freshly prepared glucanex solution (5 ml glucanex solution/plate, 10 mg glucanex / 1 ml of 0.7 M KCl) and incubate them for about 1 hour 15 minutes at room temperature. Monitor the protoplast formation in microscope. When protoplast formation is completed, wash the cellophane sheets in 100 ml 0.7 M KCl and filter the protoplast suspension through a cheese filter with 100 µm large pores. Collect the protoplasts by centrifugation at 4000 g for 25 minutes at 14 °C. Discard the supernatant and suspend the pellet by hand shaking (do not vortex). Wash the protoplasts with 10 ml of 0.7 M KCl and after collecting them by centrifugation (4000 g for 25 minutes at 14 °C) resuspend them in 1 ml 0.7 M KCl. Count the protoplasts in a haemocytometer (Burker chamber) under a light microscope. Document the calculated numbers of ptoroplasts. For protoplast fusion, mix 10^4 protoplasts of strain HZS.119 and HZS.544 into a single eppendorf tube and centrifuge the mixed protoplasts at 2500 rpm for 6 minutes. Discard the supernatant and suspend the protoplasts in 200 µl TN1 solution. Add 1 ml of TN2 solution to the sample and transfer the sample into a 15 ml large Falcon tube and add 10 ml KCl to it. Centrifuge the protoplasts at 4000g for 20 minutes at 14 °C. Suspend the protoplasts into 1 ml 0.7 M KCl by hand shaking and place the suspended protoplasts into a 50 ml Falcon tubes (or 20 cm long glass test tubes). Add 15 ml freshly prepared SMM top agar (40-42 °C) to the protoplasts, mix the sample by hand shaking and distribute the protoplast-top agar mixture on the suface of three SMM plates (about 5 ml/petri dish) in each case. Try to spread the top agar on the surface of SMM medium evenly by gently moving the plates. Incubate the plates for 4-5 days at 37 °C.

2nd week

Goal

Isolation of diploid conidiospores from the heterokaryon.

Required materials

2 pieces of G-2 sintered glass filter with 1-2 cm diameter; 2 pieces of sterile 10 ml glass centrifuge tubes; 2 pieces of half-sized sterile glass tubes filled with 5 ml sterile destilled water; 8 pieces of half-sized sterile glass tubes filled with 4.5 ml sterile destilled water, cork borer with 3 mm diameter, dissecting lancet; haematocytometer (Bürker-chambre); light microscope, stereo microscope; centrifuge; 400 ml MM (2% (v/v) salt solution, 1% (w/v) glucose, 10 mM $NaNO_3$, 2.5% (w/v) agar, pH 6.8); 400 ml CM (2% (v/v) salt solution, 1% (w/v) glucose, 2 g/l pepton, 1.5 g/l casamino acids, 1 g/l yeast extract, 2.5% (w/v) agar, pH 6.8 supplemented with multi-vitamin); 50 ml sterile destilled water; 2 pieces of sterile painting brush; 27 pieces of sterile Petri dishes, ethanol and glas streamer for streaming.

Tasks

Autoclave the MM and CM media and make 15 MM and 15 CM plates. Study the heterokaryon colonies under the stereo microscope! Isolation of diploids will be carried out by using two methodological approaches.

1. Collect conidiospores from the heterokaryotic colonies by using wet paint brush and the half-sized sterile glass tubes with 5 ml destilled water. Create a dense suspension! Eliminate the mycelial contaminations by filtering the conidiospore suspension using the G-2 sintered filter. Place the purified conidiospore suspension into a glass centrifuge tube and collect the conidiospores by centrifugation with 3000 g for 5 minutes. Discard the supernatant and re-suspend the conidiospores in sterile destilled water and adjust the conidiospore concentration to 10^6-10^7 conidiospore / ml by counting conidiospores in haematocytometer (Bürker-chambre). Stream 100 µl volumes of this conidium suspension to the surface of a MM plate in 5 replicates using the glass streamer and the sterile cabinet. Let the plates dry under the sterile cabinet before placeing the plates into the 37 °C incubator! Incubate the plates for 5-7 days!

Prepare a 4 steps series of ten times dilution from the conidiospore suspension and stream 100 µl volumes of the last two steps of the dilution series (10^{-3} and 10^{-4}) onto the surface of a CM plate in 3 replicates using the glass streamer and the sterile cabinet. Let the plates dry under the sterile cabinet before placeing the plates into the 37 °C incubator! Incubate the plates for 3 days!

2. Cut out 40 discs from different regions of the heterokaryon colony using the cork borer. Place 5 disks face down onto the surface of MM plate in five replicates and CM plates in three replicates. Incubate the plates on 37 °C for a week. On CM the heterokaryon segregates to parental clones, on MM diploid sectors might grow in case the heterokaryotic mycelia disks accidentally carried diploid compartments.

3rd week

Goal

Initiation of haploidization in the diploid colony using benomyl treatment.

Required materials

Diploid colonies; 250 ml and 100 ml CM (2% (v/v) salt solution, 1% (w/v) glucose, 2 g/l pepton, 1.5 g/l casamino acids, 1 g/l yeast extract, 2.5% (w/v) agar, pH 6.8 supplemented with multi-vitamin); 1.0 mg/ml benomyl stock solution (in DMSO); one sterile glass pot marked at 100 ml volume; pipettes with sterile tips; sterile eppendorf tubes with 500 µl Tween-80 solution; sterile tooth ticks; glass cellular debris collector.

Tasks

Make 4 Petri dish plates from autoclaved CM supplemented with 0.75 µg/ml benomyl (add 75 µl benomyl stock solution to 100 ml CM, mix it and pour 4 plates) and 4 Petri dish CM plates supplemented with 1.0 µg/ml benomyl (add 100 µl benomyl stock solution to 100 ml CM, mix it and pour 4 plates). Make two benomyl free CM plates too, as controls. Prepare a conidiospore suspension from the diploid colony in 500 µl Tween-80 containing eppendorf tube using a sterile tooth tick. Inoculate the 2 x 4 CM plates with the diploid conidiospores by placing 10 µl of the conidiospore suspension to the surface of the plate in five replicates (five spots/plate). Dry the spots under the sterile cabinet and place the plates in 37 °C incubator. Incubate the plates for 1 week.

4th week

Goal

Isolation and genetic analysis of haploid sectors segregated from the diploid colonies.

Required materials

Benomyl treated diploid colonies; 4 x 100 ml MM (2% (v/v) salt solution, 1% (w/v) glucose, 10 mM $NaNO_3$, 2.5% (w/v) agar, pH 6.8); 5 ml of different 100 x vitamin stock solutions: thiamine (thia); riboflavine (ribo) and para-amino benzoic acid (paba); 12 pieces of sterile Petri dishes; 50 sterile eppendorf tubes with 100 µl Tween-80; sterile tooth ticks; glass cellular debris collector; loop inoculator; microscope, scissors, cellux tape.

Tasks

On the benomyl supplemented CM, the diploids undergo haploidization that can be detected by the formation of withe and yellow sectors in the green colored diploid colonies. In order to study the genotype of the sectors we have to test the sectoring clones on MM containing the parental vitamin requirements in different combination. Pour 4 plates from 100 ml MM supplemented with thia, ribo, paba; 4 plates from 100 ml MM supplemented with thia, ribo; 4 plates from 100 ml MM supplemented with thia, paba; 4 plates from 100 ml MM supplemented with ribo, paba. Collect conidiospore samples from clear regions of sectors into the eppendorf tubes with 100 µl Tween-80 and inoculate the 4 differently supplemented MM plates with these conidiospore suspensions using a loop inoculator (streak a single line). Inoculate no more than 15 clones per one plate. Incubate the plates for 2-3 days at 37 °C prior reading out the growth test!

Mount a slide with conidiospore samples taken by cellux tape from green colored diploid regions and yellow or white haploid sectors and study the size difference of the diploid and haploid conidiospores under light microscope! Document the size differences by making a draw in the text book!

Determine the genotype of the sectoring clones by reading out the growth test!

Segregation of diploid created by the heterokaryon of HZS.119 and HZS.544						
Media / Strains	MM thia ribo paba	MM thia ribo	MM thia paba	MM ribo paba	color of conidia	GENOTYPE
HZS.119						
HZS.544						
diploid of HZS.119 and 544						
sector 1						
sector 2						
sector 3						
sector 4						
sector 5						
sector 6						
sector 7						
sector 8						
sector 9						
sector 10						
sector 11						
sector 12						
sector 13						
sector 14						
sector 15						
sector 16						
sector 17						
sector 18						
sector 19						
sector 20						
sector 21						
sector 22						
sector 23						
sector 24						
sector 25						
sector 26						

sector 27						
sector 28						
sector 29						
sector 30						
sector 31						
sector 32						
sector 33						
sector 34						
sector 35						
sector 36						
sector 37						
sector 38						
sector 39						
sector 40						

Did you find the sectors of being recombinants?

.....

.....

.....

.....

.....

Check your progress

After the completion of the practical course, answer the following questions.

- **What does it mean:**
  - **heterokaryon**
  - **haploid**
  - **diploid**
  - **aneuploid**
  - **chromosome non-disjunction**
  - **haploidization**
  - **mitotic (somatic) crossing over**
- **How would you isolate diploid conidiospores?**
- **How would you determine the genotype of the progeny derived from the genetic cross of two vitamin auxotroph parents?**
- **How do you explain the size difference between haploid and diploid conidiospores?**

3.2. Basic Techniques On The Field Of Microbial Ecology

3.2.1. Determination of bacterial and fungal CFU in soil samples
- Practice 18 -

Learning Objectives

After the completion of the practical course, you should be able to:

- **Working safely with soil samples**
- **Prepare serial dilution from soil samples**
- **Prepare and use of selective media for bacterial and fungal samples**
- **Purify bacteria from soil samples**
- **Purify filamentous fungi from soil samples**
- **Describe the mode of action of the antifungal compound nystatin**
- **Describe the mode of action of the antifungal compound carbendazim**
- **Describe the mode of action of the antimicrobial compound streptomycin**
- **Define the following terms: Colony Forming Unit (CFU), serial dilution, 10-fold dilution, selective medium**
- **Calculate the Colony Forming Unit (CFU) of soil samples for bacteria**
- **Calculate the Colony Forming Unit (CFU) of soil samples for fungi**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, learning sterilization techniques, usage of automatic pipettes, usage of sterile cabinets, preparation of culture media, making serial dilution from soil samples, inoculation of filamentous fungi and bacteria, culturing of filamentous fungi and bacteria on solid media**

I. Introduction

What does Colony Forming Unit (CFU) mean?

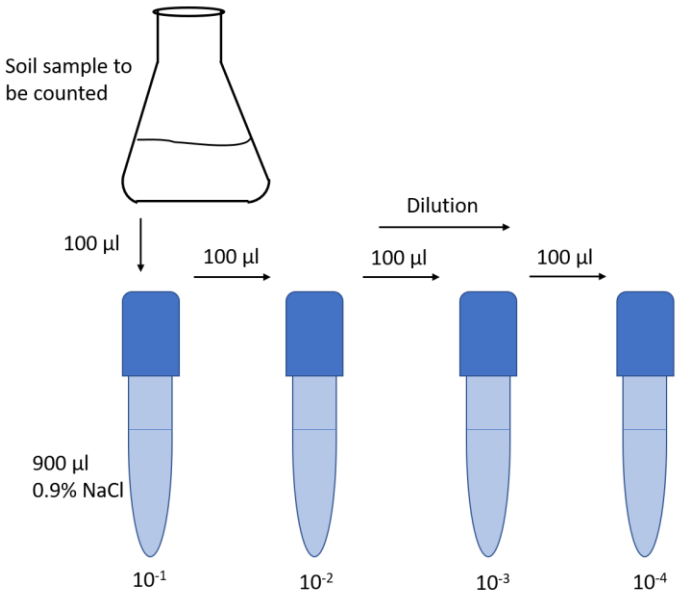
The Colony Forming Unit (CFU) is a number that corresponds to individual colonies of bacteria, yeast or filamentous fungi present in a sample. Bacterial or yeast colonies are a mass of individual cells of the same microorganism. In a colony, the individual cells are growing together. However, the growing hyphae of the same fungus refers to a colony in case of the filamentous fungi.

During this practice, CFU for bacteria and fungi in soil samples will be determined.

Since the samples may come from different surroundings, CFUs can be presented as CFU per unit volume, CFU per unit weight, or CFU per unit area.

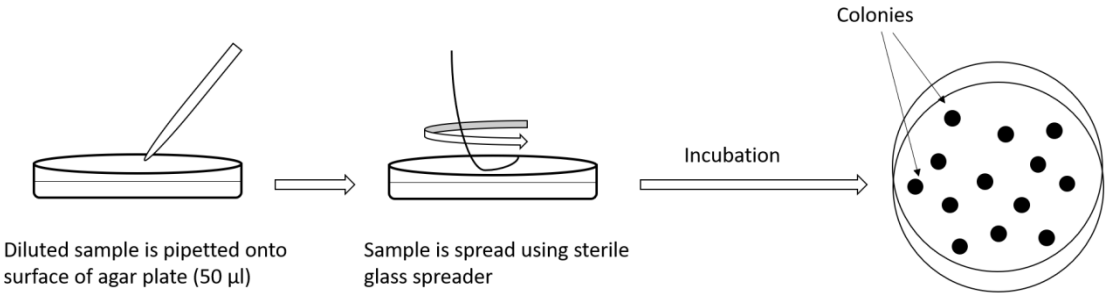
Determination of CFU from soil samples – How can I calculate it?

i) Preparation of serial dilution from soil samples:

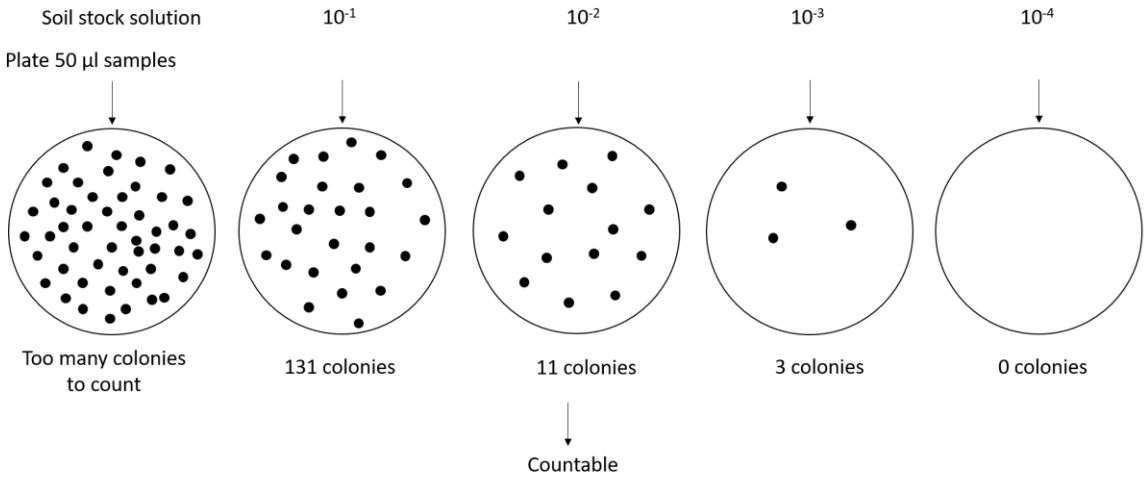


ii) Inoculation of agar plates:

A sample from each dilution step is spread on a surface of a previously prepared selective agar plate. Then, the plates are incubated at various temperatures for a number of days.



After the incubation, the colonies that form are counted:



iii) Calculation of colony forming unit

Take the plate(s) on which the colonies are countable. Then, use the following equation for the calculation:

Plate count * dilution factor = Cells (CFU) per milliliter of original sample

Because 50 µl samples will be spread from each dilution in the current practice, you must multiply the result by 20 to get the CFU per milliliter of original sample!

Selective media used during the practice

Selective medium: certain microbes can multiply on it, others do not (e.g., medium that contain antibiotics).

During the practice, nystatin and carbendazim are added to the bacterium selective medium to inhibit the growth of fungi. Streptomycin is used as antibacterial additive in the selective medium for fungi. They have different mode of action, i.e.:

Nystatin forms pores in the membrane that lead to K^+ leakage, and death of the fungus.

Carbendazym inhibits beta-tubulin polimerization and mitosis.

Streptomycin inhibits the protein sythesis of bacteria.

II. The course of Practice 18

Goal

Calculation of fungal and bacterial CFU of soil samples.

Required materials

T1 medium, T2 medium, 10 Petri dishes, soil sample, 0.9% NaCl solution, 1 glass beaker, 8 test tubes, 20-200 µl pipette, 100-1000 µl pipette, vortex, glass spreader, laboratory scale, spatula, dish with alcohol, nystatin stock solution (3 mg/ml in dimethyl sulfoxide), carbendazim stock solution (3 mg/ml in dimethyl sulfoxide), streptomycin

T1 medium: 0.5% glucose, 0.3% yeast extract, 2% agar

T2 medium: 1 % glucose, 0.3% yeast extract, 0.5% KH_2PO_4, 2% agar

Tasks

Prepare 200 ml T1 medium, autoclave it and add 1-1 ml of nystatin and carbendazim solution, respectively. Pour the prepared medium into 5 Petri dishes.
Prepare 200 ml T2 medium, autoclave it and add 20 mg of streptomycin. Pour the medium into 5 Petri dishes.
Prepare soil solution from 5 g soil and 50 ml of 0.9% NaCl. Make a series of ten-fold dilution in four steps with final volume of 1 ml using 0.9% NaCl. Spread 50-50 µl from each dilution over the surface of the appropriate selective medium. Use nystatin and carbendazim contained medium to isolate bacteria, while streptomycin contained medium to isolate fungi.
Incubate the inoculated Petri dishes at room temperature for a week.

III. Evaluation

After one-week incubation, count the bacterial and fungal colonies. Determine the living cell concentration (CFU) in the original soil sample.

CFU per unit volume (1 ml):

CFU per unit weight (1 g sample):

Check your progress

After the completion of the practical course, answer the following questions:

- **What does colony forming unit (CFU) mean?**
- **How can the colony forming unit (CFU) be determined? How is it calculated from the number of colonies?**
- **What does selective medium mean?**
- **What antibiotics have been used in the practices against bacteria and fungi?**
- **What is the mode of action of the nystatin?**
- **What is the mode of action of the carbendazim?**
- **What is the mode of action of the streptomycin?**

3.2.2. Isolation of psychrophilic, mesophilic and thermophilic bacteria from water sample
- Practice 19 -

Learning Objectives

After the completion of the practical course, you should be able to:

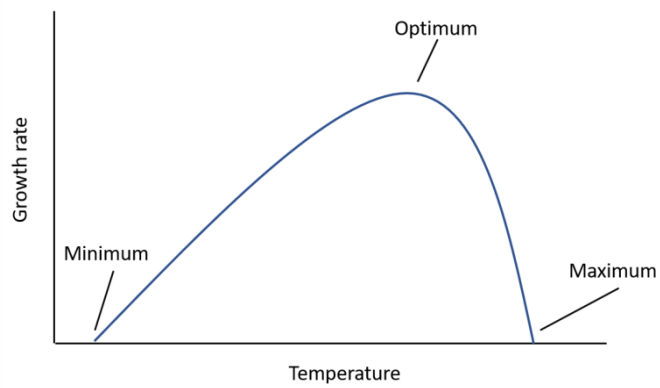
- Working safely with water samples
- Prepare serial dilution from water samples
- Prepare and use of selective media for bacterial samples
- Describe the parts of a growth curve
- Describe the effect of temperature on microbial growth
- Describe the categories of microbes based on temperature ranges of growth
- Present sample microorganisms for each categories
- List thermophilic bacteria and fungi
- List mesophilic bacteria and fungi
- List psycrophilic bacteria and fungi
- Define the following terms: Colony Forming Unit (CFU), serial dilution, 10-fold dilution, selective medium, thermophilic organism, mesophilic organism, psychrophilic organism, growth curve, minimum growth temperature, optimum growth temperature, maximum growth temperature
- Calculate the Colony Forming Unit (CFU) of water samples for bacteria
- Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, learning sterilization techniques, usage of automatic pipettes, usage of sterile cabinets, preparation of culture media, making serial dilution from water samples, inoculation of bacteria, culturing of bacteria on solid media

I. Introduction

Temperature and microbial growth

i) Growth curve

Temperature is the major limiting factor of microbial growth. Depending on the microorganisms tested, there are different minimum, optimum and maximum growth temperatures. The following figure shows an example of growth rate plotted against temperature:



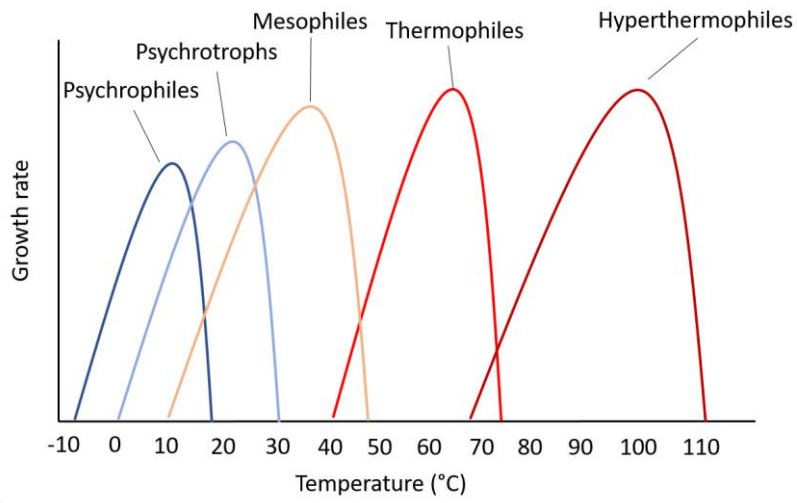
- Optimum growth temperature: the enzyme reactions work at highest catalytic rate.
- Minimum growth temperature: slow transport processes, membrane gelling, decreased enzyme activities.
- Maximum growth temperature: protein denaturation, cytoplasmic membrane collaption, DNA damage, reduced enzyme activities.

ii) Effect of temperature on the growth of microorganisms

The different temperatures affect differently the microbial metabolism. At minimal growth temperatures, the microorganism able to conduct metabolism. Under maximum growth temperature conditions, the microorganism continues a slower-rate metabolism. At optimum growth temperatures, the metabolism and thereby the growth rate are highest.

iii) Categories based on optimal growth temperature of microorganisms

The microorganisms can be categorized to different groups according to their optimum growth temperature. These are the follows:



Important to know:

Temperature tolerance of thermophilic fungi never exceed the 60 °C; at higher temperatures, eukaryotic microorganism does not occur in active form!

Obligate psychrophiles

They live in cold places; generally, they are unable to grow at room temperature conditions. The most important obligate psychrophile microorganisms, some examples:

- Bacteria: *Antarctobacter hetiothermus* (saline lake), *Colwellia rossensis* (sea ice), *Polaribacter franzmanii* (sea ice), *Pseudoalteromonas antarctica* (seawater), *Psychrobacter glacincola* (sea ice)
- Fungi: *Candida psychrophila*, *Microdochium nivale*, *Typhula incarnata*; *Typhula*: snow mold, grow under snow cover, pathogens of overwintering grasses and winter cereals

Facultative and obligate thermophiles

Habitat: hot-water areas, compost.

During composting, the temperature can rise to 60-70 °C. In the compost, the microbes are responsible for the temperature increase through their organic material metabolizing processes. These biochemical processes are exergonic and produce heat to the close environment.

The most important thermophile microorganisms in the compost, some examples:

- Bacteria: *Bacillus sp.*, *Geobacillus stearothermophilus*
- Fungi: *Rhizomucor miehei*, *Rhizomucor pusillus*, *Thermomyces lanuginosus*, *Aspergillus fumigatus*

II. The course of Practice 19

Goal

To isolate bacteria with different growth temperatures from water sample.

Required materials

T1 medium, 12 Petri dishes, water sample, 0.9% NaCl solution, 1 glass beaker, 3 test tubes, 20-200 µl pipette, 100-1000 µl pipette, vortex, glass spreader, dish with alcohol, nystatin stock solution (3 mg/ml in dimethyl sulfoxide), carbendazim stock solution (3 mg/ml in dimethyl sulfoxide)

T1 medium: 0.5% glucose, 0.3% yeast extract, 2% agar

Tasks

Autoclave 300 ml T1 medium and add 1-1 ml of nystatin and carbendazim solution. Pour the medium into 12 Petri dishes.

Make a series of ten-fold dilution in three steps with 0.9% NaCl from the water sample. Spread 50-50 µl from these dilutions, and from the initial water sample over the surface of the agar plate.

Incubate 4-4 Petri dishes at 5 °C, 35 °C and 45 °C for a week.

III. Evaluation

After one-week incubation, count the bacteria colonies on each Petri dish incubated at different temperature conditions. Compare the number of colonies obtained. Determine the CFU of the initial water sample.

Number of colonies on the plate incubated at:

5 °C (psychrophile microorganisms):

35 °C (mesophile microorganisms):

45 °C (thermophile microorganisms):

CFU per unit volume (1 ml) in the initial water sample:

Check your progress

After the completion of the practical course, answer the following questions:

- **How can microbes be categorized on the basis of temperature ranges for growth?**
- **How are the physiological processes in the microbes evolve by changing the ambient temperature?**
- **What characterizes the obligate psychrophilic microorganisms? Write two examples for fungi.**
- **What characterizes the facultative and obligate thermophilic microorganisms?**
- **What microbiological processes are taking place in the compost?**
- **How did you isolate psychrophilic, mesophilic and thermophilic bacteria during the practice?**

3.2.3. Isolation of *Pseudomonas* and *Bacillus* strains from soil samples
- Practice 20 -

Learning Objectives

After the completion of the practical course, you should be able to:

- Working safely with soil samples
- Prepare serial dilution from soil samples
- Prepare and use of selective medium to isolate *Pseudomonas*
- Describe the importance of bacteria in the soil
- Describe the basic characteristics of *Bacillus* and *Pseudomonas* bacteria
- Describe the practical importance of *Bacillus* and *Pseudomonas* bacteria
- List some important *Bacillus* and *Pseudomonas* model organisms
- Describe the differences between bacterial endospores and vegetative cells
- Describe the mode of action of trimetophrim antibiotic
- Define the following terms: Colony Forming Unit (CFU), serial dilution, 10-fold dilution, selective medium, bacterial endospore, vegetative cell, carbon cycle, nitrogen cycle, bioremediation, biocontrol
- Calculate the Colony Forming Unit (CFU) of soil sample for bacteria
- Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, learning sterilization techniques, usage of automatic pipettes, usage of sterile cabinets, preparation of culture media, preparation of agar slants, making serial dilution from soil samples, inoculation of bacteria, culturing of bacteria on solid media

I. Introduction

Why are bacteria important in soil? What is their role in the soil?

The dominant microorganisms in the soil are the bacteria. In general, they have different metabolic processes, oxygen demand (both aerob and anaerob bacteria can be found) and pH dependence (optimum pH for growth between 6.0-8.0). By function, they can be divided into five groups:

- Autotrophs – photosynthesizers: these bacteria are primary producers.
- Decomposers: catalyze the degradation of organic materials (e.g., plant residues) in the soil. They release nutrients that the plants can utilize.
- Mutualists: they can help the nutrient uptake of plants through associative relationships (e.g., nitrogen fixing bacteria).
- Pathogens: can cause various plant diseases.
- Chemolithotrophs: they obtain energy by transforming inorganic materials.

The soil bacteria are important to maintain the carbon cycle through photosynthesis and organic matter degradation (cellulose and chitin degradation, mainly by actinomycetes), and the nitrogen cycle through nitrogen fixation, denitrification and nitrification processes. In addition, the soil bacteria are capable of bioremediation as well, in which they remove the environmental pollutants or prevent soil pollution.

The genus *Pseudomonas*

The Gram-negative *Pseudomonas* species are important from many practical aspects. Within this group of microorganisms, different types of pathogen soil bacteria, potential biocontrol organisms and effective benzene, toluene and xylene degrader species can also be found. Most important *Pseudomonas* species in agriculture are the

Pseudomonas fluorescens:

- Biocontrol – can inhibit the growth of filamentous fungi and Gram-negative bacteria
- Biofilm formation,
- Quorum sensing model organism,

- Role in soil carbon and nitrogen cycles;

Pseudomonas putida:

- Several isolates with effective degradation potential against xenobiotics (e.g., isolates that can degrade benzene derivatives);

Pseudomonas aeruginosa:

- Several isolates can be utilizable as biofungicide – they reduce the growth of plant pathogenic fungi.

To isolate *Pseudomonas* bacteria from environmental samples, a selective medium that contains trimethoprim antibiotic can be used (selective medium, see Practice 18). The trimethoprim inhibits the action of dihydrofolate reductase enzyme which catalizes the formation of tetrahydrofolic acid from dihydrofolic acid. The tetrahydrofolic acid is a precursor in the thymidine synthesis pathway and thus, the inhibition of this process leads to inhibition of bacterial DNA synthesis. The trimethoprim antibiotic failure to penetrate into *Pseudomonas* cells because these bacteria have at least two efficient multiple drug efflux systems. Therefore, *Pseudomonas* cells can survive under selective conditions supported by trimethoprim.

The genus *Bacillus*

The Gram-positive rod *Bacillus* bacteria are mostly aerobic organisms. The genus contains many saprotrophic strains that can be isolated from soil, water and air samples. In soils, they are common in the plant rhizosphere as well. Some species (e.g., *Bacillus cereus*) can grow in food and cause food poisoning. The common characteristics of *Bacillus* bacteria is the formation of endospores, that structures can help the bacteria to tolerate harsh environmental conditions. The main differences between vegetative cells and endospores are the follows:

Characteristic	Endospore	Vegetative cell
Water content	Low (10-25%)	High (80-90%)
Enzymatic activity	Low	High
Metabolism (O_2 uptake)	Low or absent	High
Macromolecular synthesis	Absent	Present
mRNA	Low or absent	Present
Radiation resistance	High	Low
Heat resistance	High	Low
Resistance to chemicals	High	Low

The genus *Bacillus* contains organisms that can be used as biocontrol agents as well. For instance, strains of *Bacillus thuringiensis* and *Bacillus subtilis* are utilized as insecticide and bactericide and fungicide agents, respectively, in practical applications.

Selection method to isolate *Bacillus* bacteria:

Thanks to endospores the *Bacillus* bacteria can tolerate very high temperature conditions as well. During the practice, heating of the soil solutions at 90 °C for 15 min will provide the selection of *Bacillus* bacteria from the sample. The other microorganisms in the solutions do not tolerate this environment.

II. The course of Practice 20

Goals

1) To isolate *Pseudomonas* and *Bacillus* bacteria from soil sample using selective culturing methods.

2) To prepare agar slants that will be used during the Practices 21 and 22.

Required materials

T3 medium, T1 medium, 10 Petri dishes, soil sample, 0.9% NaCl solution, 1 glass beaker, 18 test tubes, 20-200 µl pipette, 100-1000 µl pipette, vortex, water bath, glass spreader, dish with alcohol, laboratory scale, spatula, nystatin stock solution (3 mg/ml in dimethyl sulfoxide), carbendazim stock solution (3 mg/ml in dimethyl sulfoxide), trimethoprim

T3 medium for *Pseudomonas*: 1% saccharose, 1 ml glycerol, 0.5% casamino acid, 0.1% $NaHCO_3$, 0.1% $MgSO_4$, 0.23% K_2HPO_4, 0.12% SLS (sodium lauroyl sarcosinate), 2% agar

T1 medium for *Bacillus*: 0.5% glucose, 0.3% yeast extract, 2% agar

Tasks

1) Isolation of bacteria from soil sample:

Isolation of *Pseudomonas* bacteria: Prepare 200 ml of T3 medium, autoclave it and add 4 mg of trimethoprim (0.002%). Then, pour the medium into five Petri dishes. Prepare soil solution from 5 g soil using a volume of 50 ml of 0,9% NaCl. Make a series of ten-fold dilution in four steps using 0,9% NaCl solution. Spread 50-50 µl from these dilutions, and from the initial soil solution over the surface of the agar plates.

Isolation of *Bacillus* bacteria: Prepare 200 ml of T1 medium, autoclave it and add 1-1 ml of 3 mg/ml nystatin and carbendazim stock solution. Pour the prepared medium into five Petri dishes. Prepare soil solution from 5 g soil using a volume of 50 ml 0,9% NaCl. Make a series of ten-fold dilution in four steps using 0,9% NaCl solution. Incubate the initial and diluted samples at 90 °C for 15 minutes in water bath. After the incubation, spread 50-50 µl from the diluted, and from the initial soil solutions over the surface of the prepared agar plates.

2) Preparation of agar slants:

Prepare 200 ml of T1 medium and 15 tubes with a filter paper plug. After sterilization of the medium and the plugged tubes, measure 5-5 ml of medium into the tubes. Then, slant them on the laboratory desk avoiding that the medium contacting with the paper plug. During the next practices, *Bacillus*, *Pseudomonas* and *Trichoderma* isolates will be inoculated to these agar slants.

III. Evaluation

After one-week incubation, count the *Pseudomonas* and *Bacillus* colonies and calculate their CFUs in the initial soil sample.

CFU per unit weight (1 g sample) for *Pseudomonas*:

CFU per unit weight (1 g sample) for *Bacillus*:

Check your progress

After the completion of the practical course, answer the following questions:

- **What was the procedure of *Bacillus* isolation from soil?**
- **Which characteristic of *Bacillus* bacteria can be utilized for the process?**
- **What bacteria can produce endospores?**
- **What are the main differences between bacterial endospores and vegetative cells? List five main differences.**
- **What was the procedure of *Pseudomonas* isolation from soil?**
- **What antibiotic was used for the isolation of *Pseudomonas* bacteria?**
- **What is the mode of action of trimethoprim antibiotic?**

3.2.4. Isolation of *Trichoderma* strains from soil sample
- Practice 21 -

Learning Objectives

After the completion of the practical course, you should be able to:

- **Working safely with soil samples**
- **Prepare serial dilution from soil samples**
- **Prepare and use of selective medium to isolate *Trichoderma***
- **Present the main characteristics of the genus *Trichoderma***
- **List the most important *Trichoderma* species**
- **Describe the process of *Trichoderma* mycoparasitism**
- **Define the following terms: Colony Forming Unit (CFU), selective medium, filamentous fungi, bioremediation, biocontrol, antagonism, mycoparasitism, green mold disease**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, learning sterilization techniques, usage of automatic pipettes, usage of sterile cabinets, preparation of culture media, inoculation of filamentous fungi, culturing of filamentous fungi on solid media**

I. Introduction

Trichoderma species

The main characteristics of the *Trichoderma* species:

- Imperfect fungi, sexual forms: Ascomycota, Hypocreales, Hypocreaceae, Hypocrea genus
- Common saprotrophic fungi (degradation of plant residues in the soil)
- White, green or yellow branching hyphae; the growth of hyphae can be remarkably rapid
- Intensive spore production
- Utilization of many carbon and nitrogen sources – competition for nutrients
- Secretion of antifungal compounds – antibiosis; production of inhibitors such as toxins (e.g., gliotoxin), antibiotics, alcohols and ketones
- Production of fungal cell-wall degrading enzymes (cellulases, chitinases, glucanases, proteases)
- Haustoria formation – mycoparasitism. Typical formation is the coiling which means that the parasite's hyphae grow around the host's hyphae. Then, the *Trichoderma* hyphae penetrate the host's cells, grow intracellularly in the host and obtain nutrients from it.
- The *Trichoderma* species are effective antagonist of other fungi → BIOCONTROL

In addition, certain *Trichoderma* species exhibit plant growth promoting effect which is also important feature of a biocontrol organism. Production of hormone like compounds and making available of soil nutrients for the plant are the background of the growth enhancing effect.

The most important *Trichoderma* species for biocontrol purposes are *Trichoderma harzianum*, *Trichoderma atroviride*, *Trichoderma asperellum* and *Trichoderma virens*. Several *Trichoderma* contained products are available in the market and are used in agro-industrial applications.

Worth to mentioning that some *Trichoderma* species, e.g., *Trichoderma aggressivum*, could be mushroom competitor causing severe problems (known as green mold disease) for the mushroom growers. The *Trichoderma* produces antifungal compounds in mushroom compost that inhibit mycelial growth of the commercial mushroom *Agaricus bisporus*. In Hungary, it generally causes about 60-100% damage.

II. The course of Practice 21

Goals

1) To isolate *Trichoderma* fungi from soil sample using selective culturing methods.

2) To inoculate *Bacillus* and *Pseudomonas* bacteria isolated from soil sample during Practice 20.

Required materials

1) Isolation of *Trichoderma* from soil sample:

T4 medium, 5 Petri dishes, soil sample, 5 test tubes, 20-200 µl pipette, 100-1000 µl pipette, laboratory scale, spatula, 0.2% dichloran stock solution in ethanol, 5% Rose Bengal stock solution, streptomycin

T4 medium: 1% glucose, 0.5% peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4*7H_2O$, 2% agar

The Rose Bengal and the streptomycin are required for selective isolation of fungi. Dichloran inhibits the growth of *Mucor* and *Rhizopus* strains. These fungi grow quickly, thereby, could cover the other soil fungi (e.g. *Trichoderma*) that grow slowly on the plate. This is important when isolating and purifying *Trichoderma* fungi from the soil sample.

2) Inoculation of *Bacillus* and *Pseudomonas* isolates:

Petri dishes containing *Bacillus* and *Pseudomonas* bacterial colonies isolated from soil (Practice 20), 10 T1 agar slants, inoculation loop

Tasks

1) Isolation of *Trichoderma* from soil sample:

Autoclave 300 ml of T4 medium and add 300 µl of 0.2% dichloran, 150 µl of 5% Rose Bengal and 30 mg streptomycin (0.01%). Then, pour the prepared medium into five Petri dishes. Scatter 0.5-1

g of soil sample on the surface of the solid medium and incubate the plates for a week at room temperature.

2) Inoculation of *Bacillus* and *Pseudomonas* isolates:

Pick 5-5 *Bacillus* and *Pseudomonas* colonies from the selective plates prepared during the Practice 20. Inoculate them to 5-5 T1 agar slants prepared on the Practice 20.

III. Evaluation

1) After one-week incubation, count the *Trichoderma* colonies and calculate the CFU in the initial soil sample.

Number of *Trichoderma* colonies:

CFU per unit weight (1 g sample):

2) Check the *Bacillus* and the *Pseudomonas* isolates inoculated onto T1 agar slants.

Check your progress

After the completion of the practical course, answer the following questions:

- **Which are the main characteristics of *Trichoderma* species?**
- **List three properties that responsible for the *Trichoderma* antagonism.**
- **Why are *Trichoderma* species good candidate as biocontrol organism?**
- **How does the process of *Trichoderma* mycoplasmism take place?**
- **What was the procedure of *Trichoderma* isolation from soil?**
- **What additives were used for the isolation of *Trichoderma* from soil?**
- **Why do dichloran be added to the medium?**
- **Why do Rose Bengal and streptomycin be added to the medium?**

3.2.5. Isolation of laccase producing microorganisms - Practice 22 -

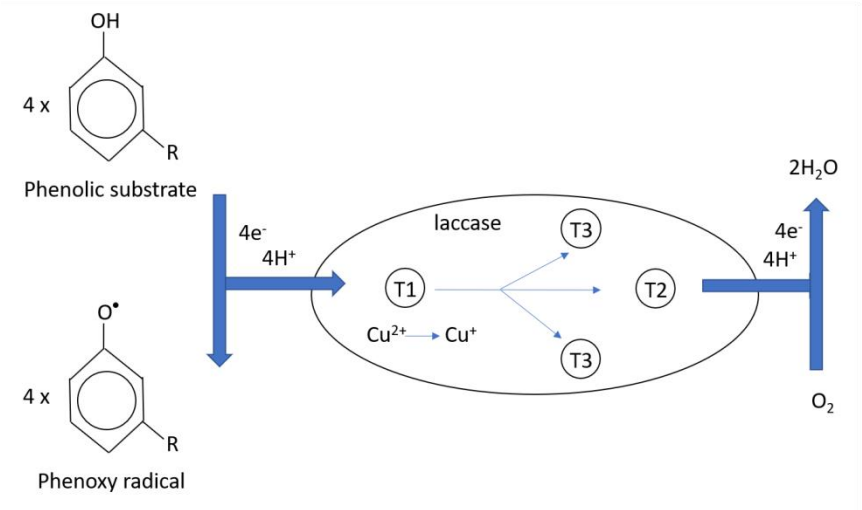
Learning Objectives

After the completion of the practical course, you should be able to:

- **Describe the basic structure of the active center of laccases**
- **Describe the reaction mechanism of laccases**
- **Prepare detection media to separate laccase producing microorganisms**
- **Separate laccase producing microorganisms from the environment**
- **Describe the background of laccase detection with 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)**
- **Describe the background of laccase detection with 2,6 dimethoxyphenol**
- **Describe the background of laccase detection with guaiacol**
- **Define the following terms: laccase, filamentous fungi, biodergadation, phenol derivatives, aniline derivatives, 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,6 dimethoxyphenol, guaiacol**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, usage of automatic pipettes, preparation of culture media, culturing of bacteria and filamentous fungi on solid media**

Laccases

Laccases are copper containing oxidases found in all higher plants, fungi, bacteria and insects. The laccases contained a total of four copper centers, which are distributed into three types designated as Type 1 (T1), Type 2 (T2) and Type 3 (T3). The difference between the types could be deduced to the wavelength of maxiumum absorbance. During the laccase catalyzed reaction, with the assistance of the four copper atoms, reduction of dioxygen to water is occurred with a concominant oxidation of diversely substituted aromatic derivatives (e.g., aromatic amines, phenolic compounds, methoxy-substituted phenols, etc.) into radicals:



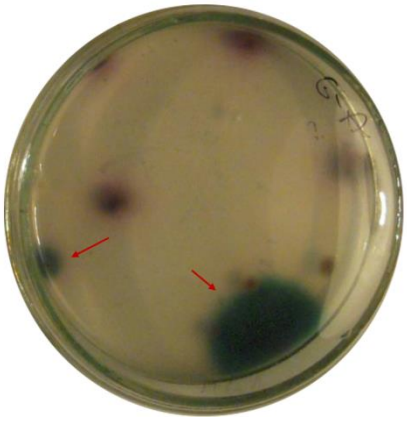
Concerning microorganisms laccases were characterized from *Bacillus subtilis*, *Azospirillum lipoferum* and different *Streptomyces*, Ascomycete and Basidiomycete species.

Isolation of laccase producing fungi on detection media

The substrates 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), guaiacol and 2,6-dimethoxyphenol can be used to detect the laccase production of microorganisms. Oxidation of theses compounds resulted in colored products around the colonies, thereby, isolates with laccase activity can easily be separated from environmental samples.

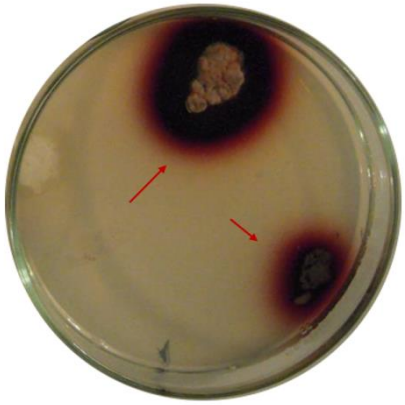
i) Detection with ABTS

Oxidation of the substrate resulted in bluish-green product around the producer colonies:



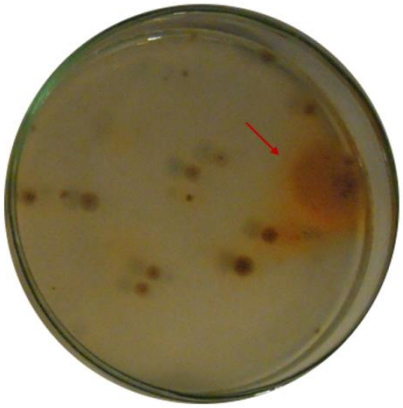
ii) Use of guaiacol for laccase detection

Guaiacol (ortho-methoxyphenol) is a non-toxic organic compound can be used for inexpensive detection of laccase production. Generally, it is applied in 1 mg/ml concentration. Oxidation of the substrate resulted in brown-red product around the producer colonies:



iii) 2,6-Dimethoxyphenol as chromogenic substrate

Oxidation of the substrate resulted in orange product around the producer colonies:



II. The course of Practice 22

Goal

To isolate laccase producer microorganisms (mainly fungi) from air using different chromogenic substrates for the detection.

Required materials

T14 medium, 3 glass bottles, 9 Petri dishes, laboratory scale, spatula, 20-200 µl pipette, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), 2,6-dimethoxyphenol, guaiacol

T14 medium: 0.5% glycerol, 0.1% KH_2PO_4, 0.1% $MgSO_4$, 0.1% $(NH_4)_2SO_4$, 0.02% $CuSO_4$, 2% agar

Tasks

Prepare three bottles of T14 medium (100-100 ml). After autoclaving, allow them cooling down to room temperature. Then, put three different chromogenic laccase substrates into the bottles: 50 mg ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) into the first bottle, 50 mg 2,6-dimethoxyphenol into the second bottle and finally 50 µl guaiacol into the third bottle. Mix the perpared media well immediately and pour them into 3-3 Petri dishes. After solidification of the medium, leave the Petri dishes in the laboratory without cover for 30 minutes. Incubate the plates under dark condition at 25 °C for 7 days.

III. Evaluation

After the incubation, detect the laccase producing isolates, count them and compare the results obtained with the different chromogenic laccase substrates.

Number of the laccase producer isolates detected with ABTS:

Number of the laccase producer isolates detected with 2,6-dimethoxyphenol:

Number of the laccase producer isolates detected with guaiacol:

Check your progress

After the completion of the practical course, answer the following questions:

- **What are laccases?**
- **What reaction is catalyzed by laccases?**
- **What is the general structure of the active center of laccase enzymes?**
- **Which microorganisms can produce laccase in high amount?**
- **List at least three potential laccase producer microorganisms.**
- **Which substrates were used for laccase detection during the practice?**
- **How did we isolate laccase producing microorganisms during the practice? Describe the used substrates and the method shortly.**

3.2.6. Analysis of *in vitro* antagonism of bacterium isolates against filamentous fungi
- Practice 23 -

Learning Objectives

After the completion of the practical course, you should be able to:

- **Describe and characterize the types of interaction between microorganisms**
- **Describe the mutually beneficial interactions**
- **Describe the antagonistic interactions**
- **Explain the mechanisms of biocontrol organisms**
- **List biofungicide and biobactericide bacteria**
- **Explain the background of the *Bacillus* antagonistic activity**
- **Explain the background of the *Pseudomonas* antagonistic activity**
- **List peptidantibiotics**
- **Working safely with plant pathogen fungi**
- **Evaluate inhibition zone data**
- **Define the following terms: population, community, biocontrol, antagonism, competition, antibiosis, biopesticide, biofungicide, biobactericide, peptidantibiotics, pseudobactin, siderophore**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, preparation of culture media, inoculation of bacteria and fungi, culturing of filamentous fungi and bacteria on solid media**

I. Introduction

Levels of ecological organization

In microorganisms, the levels of ecological organization follow the following order:

individual – population – community – ecosystem.

The complexity of interactions increases from individual to ecosystem, namely:

Individual: single organism.

Population: group of individuals of the same species that capable of interbreeding or reproduction and live in a given geographical area.

Community: assemblage of populations of different species in a given geographical area and time. Interactions can be formed between the different populations of the community.

Ecosystem: microorganism communities together with the non-living environment that interacts with them.

Interactions between populations

There are different types of interaction between the microbial populations:

Name	Effect of interaction	
	Population A	Population B
Neutralism	0	0
Commensalism	0	+
Synergism	+	+
Mutualism	+	+
Competition	-	-
Amensalism	0/+	-
Predation	+	-
Parasitism	+	-

Mutually beneficial interactions are the synergism and mutualism. In contrary, the competition, amensalism, predation and parasitism are antagonistic interactions; these relationships can be utilized for development of biopesticides.

Mechanisms of microbial biocontrol organisms

Biological control (known as biocontrol): Targeted application of one or more living organisms to the environment and use of their effect to prevent the spreading or damaging of pathogens or pests.

The mechanisms of action of microbial biocontrol organisms are:

- Antagonism: apparent reaction between organisms which could be temporal or permanent, or one-way or reciprocal.
- Direct impact on the target organism: competition, antibiosis and parasitism.
- Indirect impact on the target organism: induction of resistance against pathogens in the plant and promotion of the plant growth through liberation of nutrients in the close environment.

Application of bacteria as biocontrol agents (bactopesticides)

Bacteria for biological plant protection purposes have been researched for more than 70 years. They can be used in large quantities in a given area. The fast reproduction and the appropriate specificity against the target organism are also beneficial properties of them. In addition, the bactopesticides can be well-integrated into other defense systems as well (e.g., they are often compatible with chemical pesticides). Their antibiotic production and good competitive ability for nutrients are responsible for the antagonistic properties. Furthermore, the bactopesticides can induce resistance in the plant against plant pathogenic microbes. Several products containing different strains of *Bacillus subtilis*, *Agrobacterium radiobacter*, *Streptomyces griseoviridis*, *Pseudomonas syringae* and *Burkholderia cepacia* are available on the market.

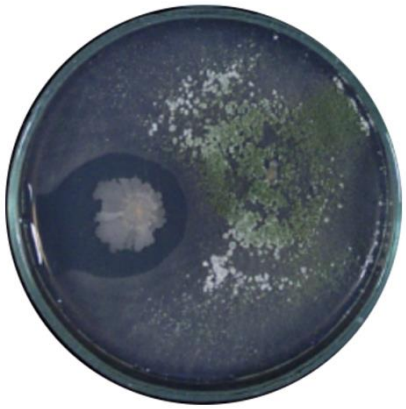
Biofungicide and biobactericide bacteria, some examples

This practice will focus on the *in vitro* inhibition of growth of plant pathogen fungi by using different *Bacillus* and *Pseudomonas* bacteria.

i) *Bacillus sp.*

The *Bacillus subtilis* is used as biopesticide in the peanut (since 1988) and cotton (since 1990) production. The target organisms are mostly *Phytophthora*, *Pythium*, *Fusarium*, *Sclerotium*, *Rhizoctonia* and *Alternaria* species. It produces peptidantibiotics such as iturin, surfactin,

fengycin, mycosubtilin and bacillomycin. *Bacillus*-based biocontrol products can be used to protect pumpkin, stone fruits, grape, soy, pea, bean, peanut, alfalfa, cotton and different other vegetables. In addition, as seen on the figure below, there are *Bacillus* isolates that can inhibit the growth of *Trichoderma aggressivum* mushroom pathogen as well:



ii) Mechanisms of biological plant protection by *Pseudomonas* species

Pseudomonas species can also be used as bactopesticides. The effect is based on the competition and the amensalism, namely:

- Competition for the iron: the bacterium produces fluorescent, low molecular weight siderophores (e.g., pseudobactin), which entrap the iron (all living organisms need iron) from the environment, thus, it will be unavailable for other microbes. However, it remained available for the plants. Antagonism against *Rhizoctonia* and *Fusarium* plant pathogens.
- Antibiosis: production of phenazine and pyrrolnitrin antibiotics. Phenazine is a wide-spectrum antibiotic that inhibits the respiration of the target microorganism.

There are many examples of biocontrol strains isolated from pseudomonads. For example, certain strains of *Pseudomonas fluorescens*, *Pseudomonas aureofaciens* and *Pseudomonas putida* species can be used against *Sclerotinia*, *Rhizoctonia*, *Pythium* or *Phytophthora* plant pathogen fungi.

II. The course of Practice 23

Goals

1) To investigate the *in vitro* inhibition of *Fusarium oxysporum*, *Rhizoctonia solani* and *Trichoderma aggressivum* filamentous fungal strains by *Bacillus* and *Pseudomonas* bacteria isolated from soil sample during Practice 20.
2) To inoculate *Trichoderma* fungi isolated from soil sample during Practice 21.

Required materials

1) *In vitro* antagonistic activity tests:
T1 medium, 6 Petri dishes, inoculation loop

T1 medium: 0.5% glucose, 0.3% yeast extract, 2% agar

Microorganisms: 5 *Bacillus* isolated from soil and maintained on T1 agar slants, 5 *Pseudomonas* isolated from soil and maintained on T1 agar slants, *Fusarium oxysporum*, *Rhizoctonia solani*, *Trichoderma aggressivum*

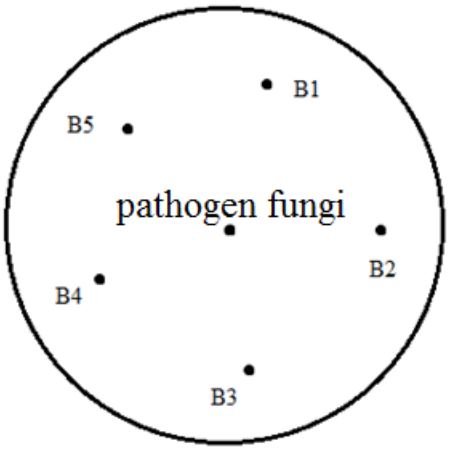
2) Inoculation of *Trichoderma* isolates:
Petri dishes containing *Trichoderma* fungal colonies isolated from soil (Practice 21), 5 T1 agar slants, inoculation loop

Tasks

1) *In vitro* antagonistic activity tests:
Pour T1 media into 6 Petri dishes. Inoculate the *Fusarium oxysporum*, *Rhizoctonia solani* and *Trichoderma aggressivum* to the center of the plates using inoculation loop. Inoculate the bacteria 3 cm far from the test fungus by using an inoculation loop. Pay attention: Sterilize the

loop between inoculation of each bacterium. In these tests, the 5 *Bacillus* and 5 *Pseudomonas* bacteria isolated from soil sample must be tested.

Follow the layout below to inoculate the fungus and the bacteria to be tested:



2) Inoculation of *Trichoderma* isolates:

Pick 5 *Trichoderma* colonies from the selective plates prepared during the Practice 21. Inoculate them to 5 T1 agar slants prepared on the Practice 20.

III. Evaluation

1) Check the *in vitro* antagonistic capacity of the *Bacillus* and *Pseudomonas* isolates against *Fusarium oxysporum*, *Rhizoctonia solani* and *Trichoderma aggressivum*.

Fill the table using the symbols indicated below:

	F. oxysporum	***R. solani***	***T. aggressivum***
Bacillus 1			
Bacillus 2			
Bacillus 3			
Bacillus 4			
Bacillus 5			
Pseudomonas 1			
Pseudomonas 2			
Pseudomonas 3			
Pseudomonas 4			
Pseudomonas 5			

Sign the antagonistic capacity with:

+++ good

++ moderate

+ weak

- no activity

2) Check the *Trichoderma* isolates inoculated onto T1 agar slants.

Check your progress

After the completion of the practical course, answer the following questions and perform the tasks indicated below:

- **List the interactions between populations. Which of these are antagonistic interactions? What is the significance of these antagonistic interactions?**
- **What are the mechanisms of microbial biocontrol organisms?**
- **Describe shortly the mechanisms of biological plant protection by *Pseudomonas* species.**
- **What mechanism is the biopesticidal activity of *Bacillus subtilis* based on?**
- **List 3 microorganisms that can be utilized for biocontrol purposes.**
- **What are siderophores? What is their role in the *Pseudomonas* biological control?**
- **What are the two interaction mechanisms that the *Pseudomonas* biocontrol based on?**

3.2.7. Fungicide tolerance of fungi
- Practice 24 -

Learning Objectives

After the completion of the practical course, you should be able to:

- **Working safely with fungicide compounds**
- **Characterize the types of fungicides in terms of their uptake**
- **Explain the mode of action of carbendazim**
- **Define the following terms: pesticide, fungicide, biofungicide**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, safe work in a microbiological laboratory, preparation of culture media, preparation of solid medium containing different concentration of pesticide, inoculation of fungi, culturing of filamentous fungi**

I. Introduction

Fungicides

Fungicides are chemical compounds (pesticides) or biological organisms (biopesticides, see Practice 23) that can be used to kill or inhibit the growth of fungi or fungal spores. Fungicides are frequently used in the agriculture and to eliminate fungal infections in animals. Most commercial fungicides are available in liquid form.

According to the take up into the plant tissues and the localization of the effect, three types of fungicides are known:

- Contact fungicides: localized effect; the fungicide compound acts directly at the site of treatment (e.g., sprayed leaf surface). The fungicide compound does not enter to the plant's tissues.
- Translaminar fungicides: the compounds distribute from the sprayed leaf surface (generally the upper side) to the unsprayed one (lower surface). The fungicide compound enters to the plant's tissues.
- Systemic fungicides: the fungicide compound enters and distributes through the xylem vessels.

Carbendazim - Mode of action

Inhibitory effect of the pesticide carbendazim will be tested during the practice. This benzimidazole fungicide has been introduced in 1976. Due to its broad spectrum, the carbendazim can be used for growth inhibition of many kinds of fungi. For instance, it is very effective against soil filamentous fungi such as *Fusarium*, *Aspergillus* and *Verticillium* species. Also, this pesticide can control the apple scab and powdery mildew diseases as well as different symptoms caused by *Botrytis* and *Gloeosporium* fungi. In these targets, the carbendazim inhibits beta tubulin polymerization and mitosis (cell division) via tubulin-benzimidazole-interactions.

Fungicide tolerance

Tolerance to fungicides is a very important property for a good biocontrol microorganism. The biofungicide can be used in combination with the chemical fungicide to which tolerance has been shown. Thus, their effects can complement each other.

II. The course of Practice 24

Goal

To investigate the fungicide tolerance of *Trichoderma* fungi isolated from soil sample during Practice 21.

Required materials

T15 medium, 8 Petri dishes, 4 mg/ml carbendazim stock solution, 6 test tubes, glass beaker, 20-200 µl pipette, 100-1000 µl pipette, inoculation loop

T15 medium: 0.5% glucose, 0.2% yeast extract, 2% agar, 5 µg/ml digitonin

Microorganisms: 5 *Trichoderma* isolated from soil and maintained on T1 agar slants

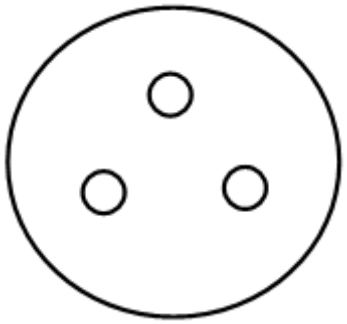
Tasks

Prepare a series of two-fold dilution from 4 mg/ml carbendazim stock solution in six steps using dimethyl sulfoxide. The final volume should be 1 ml.

Prepare 300 ml of T15 media, then, follow the procedure below to set the dilutions:
1: Pour 20 ml medium into a sterile beaker, then, pour this medium into a Petri dish.
2: Pour 20 ml medium into a sterile beaker and add 100 µl carbendazim solution (4 mg/ml stock solution), then, pour this medium into a Petri dish (the final cc. is: 20 mg/l carbendazim).
3: Pour 20 ml medium into a sterile beaker and add 100 µl carbendazim solution (2 mg/ml stock solution), then, pour this medium into a Petri dish (the final cc. is: 10 mg/l carbendazim).
4: Pour 20 ml medium into a sterile beaker and add 100 µl carbendazim solution (1 mg/ml stock solution), then, pour this medium into a Petri dish (the final cc. is: 5 mg/l carbendazim).

5: Pour 20 ml medium into a sterile beaker and add 100 µl carbendazim solution (0.5 mg/ml stock solution), then, pour this medium into a Petri dish (the final cc. is: 2.5 mg/l carbendazim).
6: Pour 20 ml medium into a sterile beaker and add 100 µl carbendazim solution (0.25 mg/ml stock solution), then, pour this medium into a Petri dish (the final cc. is: 1.25 mg/l carbendazim).
7: Pour 20 ml medium into a sterile beaker and add 100 µl carbendazim solution (0.125 mg/ml stock solution), then, pour this medium into a Petri dish (the final cc. is: 0.625 mg/l carbendazim).
8: Pour 20 ml medium into a sterile beaker and add 100 µl carbendazim solution (0.0625 mg/ml stock solution), then, pour this medium into a Petri dish (the final cc. is: 0.3125 mg/l carbendazim).

Inoculate the prepared dishes with three *Trichoderma* fungi isolated from soil during Practice 21 and maintained on agar slants. Inoculate three fungi to one Petri dish in a triangular form using inoculation loop.
Follow the layout below to inoculate the fungi to be tested:



Incubate the dishes at room temperature for a week.

III. Evaluation

After the incubation period detect the fungicide tolerance by measuring the diameters of the colonies. Fill the table:

	Colony diameter (in mm)		
Concentration of carbendazim	***Trichoderma* 1**	***Trichoderma* 2**	***Trichoderma* 3**
0 mg/ml			
0.3125 mg/l			
0.625 mg/l			
1.25 mg/l			
2.5 mg/l			
5 mg/l			
10 mg/l			
20 mg/l			

Check your progress

After the completion of the practical course, answer the following questions and perform the tasks indicated below:

- **What are pesticides?**
- **What are fungicides?**
- **How can we determine the fungicide tolerance of a fungal isolate? Write a method for this.**
- **Define carbendazim.**
- **What is the mode of action of carbendazim?**

3.3. Studies On Microbial Exoenzymes

3.3.1. Lipase production of microorganisms

Overview of the course

1. Detection of extracellular lipase activity – Practice 25
2. Measurement of lipase hydrolytic activity – Practice 26
3. Measurement of lipase synthetic activity – Practice 27

Learning Objectives

After the completion of the practical courses, you should be able to:

- **Describe the lipase catalyzed reactions**
- **List industrially important lipase producer microorganisms**
- **Detect the lipase production of microorganisms**
- **Screen lipase producer microorganisms**
- **Determine the hydrolytic activity of lipase preparations**
- **Use chromogenic substrate for enzyme activity determination**
- **Explain the benefits of *p*-nitrophenol-based substrates**
- **Working safely with organic solutions**
- **Define the following terms: extracellular enzyme (exoenzyme), fermentation, submerged fermentation, solid-state fermentation, hydrolase, lipase, triacylglycerol, fatty acid, tributyrin, hydrolytic activity, lipolytic activity, synthetic activity, esterification, transesterification, alcoholysis, chromogenic substrate**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, preparation of tributyrin contained culture media, culturing of filamentous fungi and bacteria, sampling from submerged culture, preparation of crude enzyme extract, preparation of reaction mixture for enzyme activity test**

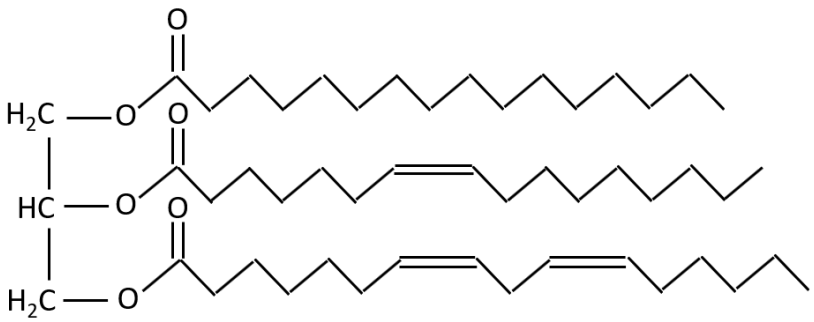
Fats and oils

Fats and oils are mixture of triacylglycerols. In chemical point of view, these are esters formed from a glycerol backbone and different fatty acids through condensation (esterification, water release). Their fatty acids are varied; and fatty acids with different chain-length and saturation can be found in the same triglyceride. In natural fats, even-numbered carbon atoms constitute the fatty acid part, which is attributed to the characteristics of their catalyzing enzyme (Acetyl-Coenzyme A). In most cases, the carbon chain has 16 or 18 carbon atoms, but it can be occurred between 4 and 24. It is important that they are always straight, unbranched chains.

Triacylglycerols (triglycerides)

Simple lipids; consists of glycerol and three fatty acids (tri- + glyceride). Carbon chains in the acid part can be unsaturated at 1x, 2x, 3x or 4x as well. The more double bonds, the lower the congelation point (solid state). The shorter chain, the lower the congelation point (solid state).

Example of triglyceride molecule. The upper fatty acid part is saturated, the middle and lower fatty acids are unsaturated at 1 and 2 times, respectively:



Natural fatty acids, some examples

i) Saturated fatty acids:

- palmitic acid: CH3–(CH2)14–COOH
- stearic acid: CH3–(CH2)16–COOH
- butyric acid: CH3–(CH2)2–COOH

- lauric acid: CH3–(CH2)10–COOH
- myristic acid (tetradecanoic acid): CH3-(CH2)12-COOH

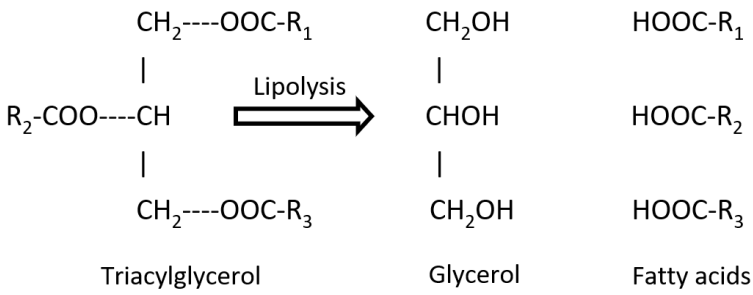
ii) Unsaturated fatty acids:

- oleic acid: CH3–(CH2)7–CH=CH–(CH2)7–COOH
- linoleic acid (omega-6 fatty acid): CH3-(CH2)4-CH=CH-CH2-CH=CH-(CH2)7-COOH
- alpha-linolenic acid, (omega-3 fatty acid): CH3-CH2-CH=CH-CH2CH=CH-CH2-CH=CH-(CH2)7-COOH
- arachidonic acid (omega 6 fatty acid): CH3-(CH2)4-CH=CH-CH2-CH=CH-CH2-CH=CH-CH2-CH=CH-(CH2)3-COOH

Lipases

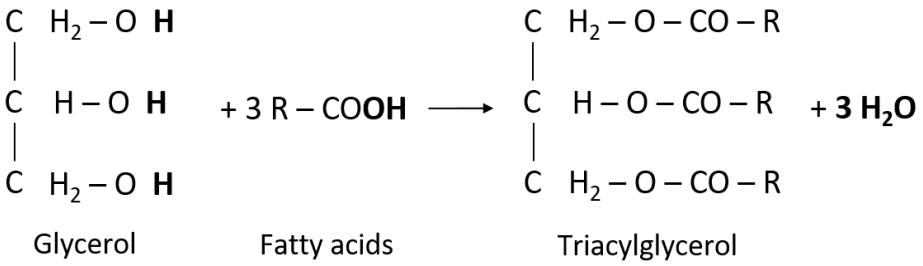
Lipases (E.C. 3.1.1.3) catalyze both the hydrolysis (lipolysis) and the synthesis (synthetic activity) of triacylglycerols. There are many good lipase producer organisms among microbes. These microbial lipases find promising applications in a wide range of biotechnological and industrial processes including flavour enhancement in food processing, biodiesel production and pharmaceutical processing. Enzyme activities of lipases in detail:

- Hydrolytic activity (lipolysis) – lipases hydrolyze the triglycerides to glycerol and fatty acid. Glycerol and fatty acid are valuable carbon sources for the microbes.



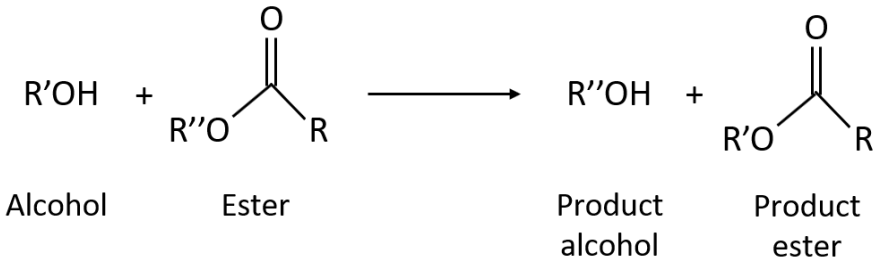
Lipolytic action of lipases

- Synthetic activity – under certain conditions, lipases can synthesize ester compounds through the catalysis of esterification, transesterification or interesterification processes. The **esterification** reaction may result in monoglycerides and diglycerides as well. Mono and diglycerides of fatty acids can be used as food additives (e.g., in margarine, chocolate, cream and meat products).



Esterification by lipases. Three molecules of water are released during the synthesis of one molecule of triacylglycerol.

The lipase catalyzed **transesterification** reaction mainly occurred under low water activity conditions. Through the reaction, translocation of ester linkages between two triacylglycerol molecules (ester exchange) or between a triacylglycerol compound and an alcohol (alcoholysis) is catalyzed. Thus, lipid molecules can be modified or new lipids compounds (e.g., alkyl-esters) can be produced by the transesterification.



The process of alcoholysis. Exchange of ester R'' group to alcohol R' group.

The esterification and transesterification reactions can be utilized in several biotechnological processes such as the synthesis of aroma-, phenyl-, alkyl esters and different polymers, pharmaceutical raw materials, fine chemicals and food additives. Transesterification processes are important in the biodiesel industry as well. Namely, diesel fuel can be produced from plant and animal fats by transesterification with methanol or ethanol.

Lipase producer microbes, some examples

Fungi

Aspergillus niger, *Candida cylindracea*, *Humicola lanuginosa*, *Rhizomucor miehei*, *Rhizopus arrhizus*, *Rhizopus delemar*, *Rhizopus japonicus*, *Rhizopus niveus*, *Rhizopus oryzae*, *Mucor* sp., *Mortierella* sp.

Bacteria

Achromobacter sp., *Alcaligenes* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Chromobacterium* sp.

Lipase detection with tributyrin

Tributyrin

- It is an ester composed of butyric acid and glycerol
- Inexpensive substrate
- It is used for simultaneous testing of large number of isolates

Lipase detection using tributyrin

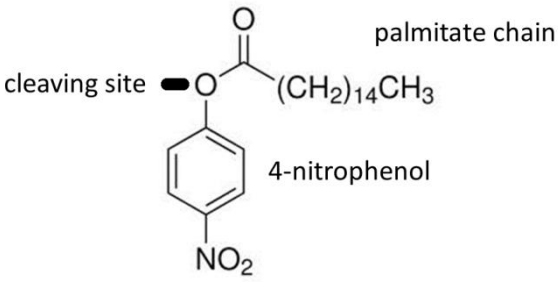
Mix the tributyrin compound with the appropriate growth medium. After solidification, the medium is opalescent. If lipase catalyzed hydrolysis of the tributyrin is occurred, a clear zone can be seen around the producer colonies. Thus, the lipase production ability of the microorganism can be estimated.

Detection of lipase production through tributyrin hydrolysis. A dergradation zone (clear) is formed around the producer colonies (red arrow).

Lipase activity measurements with chromogenic substrates

Hydrolytic activity

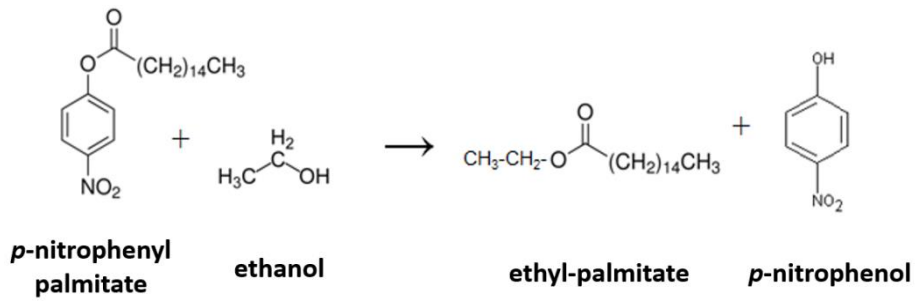
Aryl substrates having more that 10-carbon chain length fatty acid can be used safely for lipolytic activity measurements (other esterase does not disturb the reaction). Such substrates could be the 4-nitrophenyl-palmitate (C16), 4-nitrophenyl-dodecanoate (C12) etc. Background of the reaction (C16 substrate):



The lipase hydrolyzes the ester bond of the substrate (cleavage site), thereby, the chromophoric compound (4-nitrophenol) releases causing a yellow color change in the reaction mixture. The dissolved chromophore-fatty acid complex is colorless, while the free 4-nitrophenol has the yellow color which can be monitored at 405-410 nm using microplate reader. The intensitiy of the yellow color is proportional to the lipase activity in the reaction mixture. Alternatively, a well-prepared calibration curve for the 4-nitrophenol allow the calculation of enzyme activity units (U).

Transesterification activity

By means of aryl ester substrates, transesterification activity of lipases can also be determined. As with lipolytic activity measurements, the spectrophotometric method utilizes the color change caused by the liberation of 4-nitrophenol compounds. In this well-validated technique, the palmitic acid group of the 4-nitrophenyl palmitate translocates to the acyl acceptor alcohol as a result of the enzymatic catalysis. The resulting products are ethyl palmitate ester and 4-nitrophenol, which are liberated via alcoholysis. The background of the reaction is (C16 substrate):



Putative model of transesterification reaction between 4-nitrophenyl palmitate as acyl donor and ethanol as acyl acceptor.

3.3.1.1. Detection of extracellular lipase activity - Practice 25 -

Goal

To investigate the lipase production of *Bacillus* and *Pseudomonas* bacteria isolated from soil sample during Practice 20.

Required materials

T6 medium, 2 Petri dishes, laboratory glass bottle with screw cap, inoculation loop, 100-1000 µl pipette, tributyrin

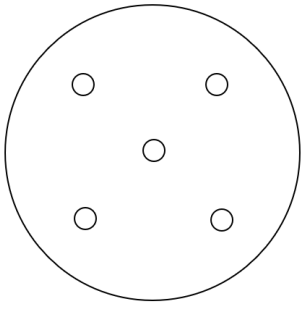
T6 medium: 0.2% yeast extract, 2% agar

Microorganisms: 5 *Bacillus* isolated from soil and maintained on T1 agar slants, 5 *Pseudomonas* isolated from soil and maintained on T1 agar slants

Tasks

Prepare 50 ml of T6 medium in a capped glass bottle. After autoclave sterilization, cool down the medium to room temperature and add 500 µl of tributyrin (1%). Then, shake the medium intensively and pour it into two Petri dishes. After the medium has solidified, inoculate the prepared dishes with 5 *Bacillus* and 5 *Pseudomonas* strains isolated from soil during Practice 20 and maintained on agar slants (5 isolates/1 Petri dish). Use inoculation loop.

Follow the layout below to inoculate the bacteria to be tested:



Then, incubate the dishes at room temperature for a week.

Evaluation

After the incubation period, measure the width of the tributyrin degradation halo around the bacterial colonies. Fill the table below with the data in millimeters:

Isolates	**Width of tributyrin degradation halo (mm)**
Bacillus 1	
Bacillus 2	
Bacillus 3	
Bacillus 4	
Bacillus 5	
Pseudomonas 1	
Pseudomonas 2	
Pseudomonas 3	
Pseudomonas 4	
Pseudomonas 5	

3.3.1.2. Measurement of lipase hydrolytic activity - Practice 26 -

Goal

To investigate and compare of the lipolytic activity of *Trichoderma* strains cultivated under submerged condition.

Required materials

150 ml inductive broth, 7 100-ml Erlenmeyer flasks, measuring cylinder, 14 Eppendorf tubes, 20-200 µl pipette, 100-1000 µl pipette, centrifuge, 96-well microtiter plate, spectrophotometer (with microplate reader function), 4 mg/ml (3 mM) *p*-nitrophenyl palmitate stock solution in dimethyl sulfoxide, 100 mM sodium phosphate buffer (pH = 6.8), 10% sodium carbonate solution

Inductive broth: 1% mannitol, 2% wheat bran, 0.5 % KH_2PO_4, 0.2% $NaNO_3$, 0.1% $MgSO_4$
Sodium phosphate buffer (100 mM, pH = 6.8): 49% 100 mM Na_2HPO_4 solution, 51% 100 mM NaH_2PO_4 solution
Sodium carbonate solution (10%): 0.5 g anhydrous sodium carbonate in 5 ml distilled water

Microorganisms: *Trichoderma harzianum* T66, *Trichoderma viride* T114, *Trichoderma atroviride* T122, *Trichoderma viride* T228, *Trichoderma harzianum* T334, *Trichoderma harzianum* T415

Tasks

Inoculation:
Measure 20 ml inductive broth per Erlenmeyer flask and inoculate them with 10 µl from *Trichoderma* suspension; 1 strain/1 flask. Leave one flask as background control (microorganism-free medium). Incubate the flasks for a week at 30 °C.

Sample preparation:

After the incubation, pipette 1-1 ml from the broths into Eppendorf tubes. Centrifuge them at 10.000 rpm for 5 minutes and transfer the supernatant to a new Eppendorf tube. This clear supernatant will be used for enzyme activity measurements.

Lipase activity measurement:

Pipette 500 µl of sodium phosphate buffer to 500 µl 4-nitrophenyl palmitate stock solution. Then, add 50 µl of buffered substrate to 50 µl supernatant in 96-well microtiter plate. After 30 min of incubation at 30 °C, add 100 µl 10% sodium carbonate solution to each tube and monitor the *p*-nitrophenol release (yellow color) at 405 nm using microplate reader.

Evaluation

Visualize the lipase activity of the *Trichoderma* isolates tested using bar graph.

Calculation: OD corresponds to enzyme activity = OD of the sample - OD of the background control.

3.3.1.3. Measurement of lipase synthetic activity - Practice 27 -

Goal

To analyze and compare of the synthetic activity of filamentous fungal lipases produced under solid-state fermentation.

Required materials

Wheat bran, distilled water, gauze, 10 100-ml Erlenmeyer flasks, 20 1.5-ml Eppendorf tubes, 10 2-ml Eppendorf tubes, 20-200 µl pipette, 100-1000 µl pipette, centrifuge, vortex, spectrophotometer, couvette, *p*-nitrophenyl palmitate, water free *n*-heptane, absolute ethanol (99.8 or 100%), 10% sodium carbonate solution, sodium sulfate

Preparation of water-free *n*-heptane: To avoid undesired hydrolysis, traces of water can be removed by using sodium sulfate. One spoon of sodium sulfate was added to 10 ml *n*-heptane solution. After intensive vortex, let the salt to settle, then, remove the supernatant from the salt by pipetting.

Sodium carbonate solution (10%): 0.5 g anhydrous sodium carbonate in 5 ml distilled water

Microorganisms: *Mucor corticolus*, *Rhizomucor miehei* (R8), *Rhizopus oryzae* (Rh26), *Rhizopus oryzae* (Rh29), *Rhizopus stolonifer, Umbelopsis autotrophica, Umbelopsis ramanniana, Umbelopsis isabellina, Mortierella alpina, Mortierella echinosphaera*

Tasks

Inoculation:

Measure 5-5 g of wheat bran to the Erlenmeyer flasks and moisturize them with 5-5 ml of distilled water. Inoculate the medium with 100 µl from spore suspension; 1 strain/1 flask. Incubate the flasks for a week at 30 °C for *Rhizomucor* and *Rhizopus* isolates and at 25 °C for *Umbelopsis* and *Mortierella* strains.

Sample preparation:

After the fermentation, add 30 ml of distilled water to the ferment and then filtrate the crude extracts through gauze. Pipette 1-1 ml from the crude extracts into Eppendorf tubes. Centrifuge them at 10.000 rpm for 5 minutes and transfer 300 µl of clear supernatant to a new Eppendorf tube. After lyophilization, the water-free crude enzyme extracts will be used for enzyme activity measurements.

Determination of lipase synthetic activity:

Suspend the lyophilized extract in 450 µl *n*-heptane containing 10 mM *p*-nitrophenyl palmitate. (Use pipette tip to scrape/suspend the dried enzyme! It will not dissolve completely!) Then, add 50 µl of absolute ethanol and incubate the reaction mixture for 60 min at 37-40 °C. After the incubation, pipette 200 µl from the reaction mixtures to a new 2-ml Eppendorf tube. Add approx. 2 ml of 10% sodium carbonate (fill the tube). Thus, the reaction is stopped and the released *p*-nitrophenol is precipitated. Finally, pour the solution to a cuvette and measure the OD at 405 nm.

Use the following solution as blank: 450 µl n-heptane containing *p*-nitrophenyl palmitate, 50 µl absolute ethanol. Measure 200 µl from this solution and add approx. 2 ml of 10% sodium carbonate solution.

Background control:

A range of lyophilized extracts should be suspended only in 500 µl *n*-heptane containing *p*-nitrophenyl palmitate (in the absence of ethanol). Scrape/suspend the dried enzyme and incubate the mixture for 60 min at 37-40 °C. After the incubation, pipette 200 µl to a new 2-ml Eppendorf tube. Add approx. 2 ml of 10% sodium carbonate (fill the tube). Pour the solution to a cuvette and measure the OD at 405 nm.

Evaluation

Visualize the synthetic activity of the tested filamentous fungal lipases using bar graph.

Calculation: OD corresponds to enzyme activity = OD of the sample - OD of the background control.

Check your progress

After the completion of the practical courses, answer the following questions and perform the tasks indicated below:

- **What are triacylglycerols? What components consist these molecules?**
- **What enzymes can degrade the triacylglycerol molecules?**
- **How do lipase enzymes work?**
- **How is lipase-catalyzed triglyceride hydrolysis? What are the reaction products?**
- **Why could the tributyrin be used to detect the lipolytic activity of microorganisms?**
- **What is the principle of the lipase-catalyzed *p*-nitrophenyl ester hydrolysis?**
- **How did we perform lipase detection during the practice? Describe the used substrate and the method shortly.**
- **How did we measure the hydrolytic and synthetic activity of lipases? Describe the used substrate and the methods shortly.**
- **Why could the *p*-nitrophenyl esters be used to test the synthetic activity of lipases?**

3.3.2. Chitinase production of microorganisms

Overview of the course

1. Detection of extracellular chitinase activity – Practice 28

2. Measurement of chitinase activity – Practice 29

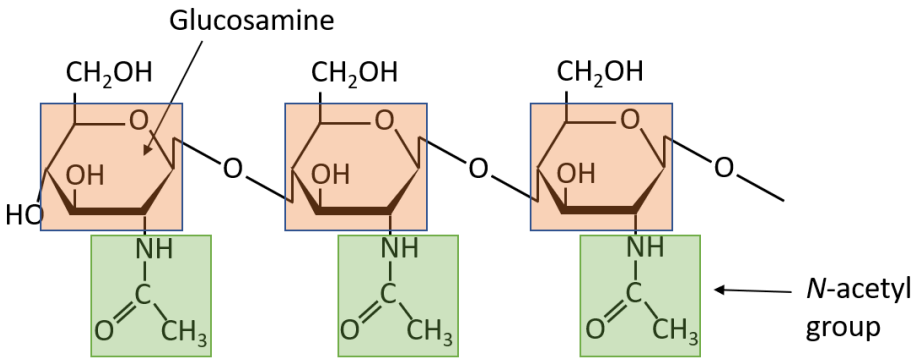
Learning Objectives

After the completion of the practical courses, you should be able to:

- **Describe the action of chitinase enzymes**
- **List the enzymes of the chitinase enzyme complex**
- **List industrially important chitinase producer microorganisms**
- **Detect the chitinase production of microorganisms**
- **Screen chitinolytic microorganisms**
- **Determine the hydrolytic activity of chitinase preparations**
- **Use chromogenic and fluorogenic substrates for enzyme activity determination**
- **Explain the benefits of *p*-nitrophenol-based substrates**
- **Working safely with organic solutions**
- **Define the following terms: extracellular enzyme (exoenzyme), submerged fermentation, hydrolase, chitinase, endochitinase, exochitinase, chitin, N-acetyl glucosamine, hydrolytic activity, chitinolytic activity, chromogenic substrate**
- **Acquire the following laboratory skills: sterile work in microbiological laboratory, preparation of colloidal chitin contained culture media, culturing of filamentous fungi and bacteria, sampling from submerged culture, preparation of crude enzyme extract, preparation of reaction mixture for enzyme activity test**

Structure of chitin

The chitin consists of N-acetyl-glucosamine subunits that linked with β (1,4) bonds forming a homopolymer polysaccharide:

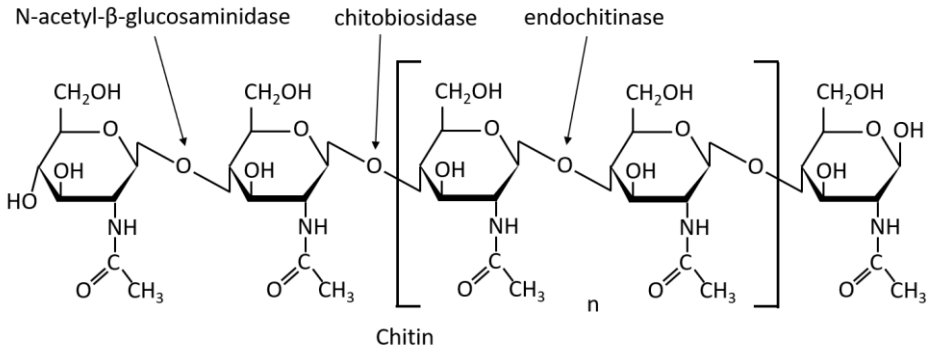


Polymer of β-(1,4)-N-acetyl-D-glucosamine units

Chitin is found in the cell wall of certain fungi and in the exoskeleton structure of insects. The annual production of this polymer is almost equal to cellulose, that indicates the presence of large amount of chitinous waste in the environment. The chitin is a biodegradable molecule, and the decomposing microbes utilize the N-acetyl-glucosamine homopolymer structure as C- and N-source.

The chitinase enzyme complex

The chitinase enzyme complex hydrolyze the chitin polymer to N-acetyl-glucosamine monomers. There are two major types of chitinase activities in this extensively studied multienzyme complex: the endochitinases and the exochitinases. The endochitinases (E.C 3.2.1.14) split randomly in the chitin chain and releases low molecular weight end-products. The exochitinases cleave N-acetyl-glucosamine (N-acetyl-β-glucosaminidases, E.C. 3.2.1.30) or chitobiose (chitobiosidases) units from the non-reducing end of the chain.



Cleavage sites of different chitinolytic enzymes

Chitinases are important in different biotechnological applications due to their ability to degrade chitin in the fungal cell wall and insect exoskeleton. This ability offers various applications of these enzymes including their use as antimicrobial or insecticidal agents. The use of chitinolytic enzymes is an important factor in the processing of chitinous wastes as well.

Chitinase producer microbes, some examples

Bacteria

The soil bacteria *Bacillus* sp., *Serratia* sp., *Pseudomonas* sp., *Streptomyces* sp., and *Aeromonas* sp.

Fungi

Aspergillus niger, *Aspergillus fumigatus*, *Trichoderma* sp., *Penicillium funiculosum*

Detection of citinase production

The chitinase production of microorganisms can be detected my adding colloidal chitin to the culture medium. The chitinolytic microorganisms degrade the substrate from their environment forming a clear halo around the colonies. The with of this halo is proportional to the chitinolytic enzyme production of the microbe.

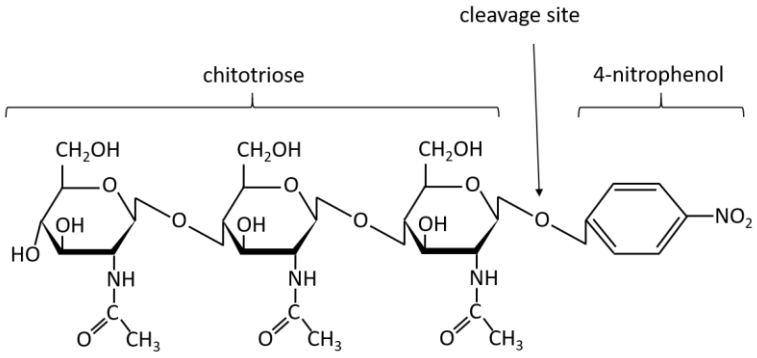
Chromogenic and fluorogenic chitinase substrates

Specific chromogenic and fluorogenic substrates can be used for the selective measurement of each activities of the chitinase enzyme system. These are synthetic 4-nitrophenol (chromogenic) or 4-methylumbelliferol (fluorogenic) based substrates and each of them are commercially

available. The chitinase cleaves the bond between the 4-nitrophenol/4-methylumbelliferol and saccharide compounds resulting in free chromophore or fluorophore molecules that cause color or fluorescence intensity change, respectively, in the reaction mixture. Both can be monitored spectrophotometrically, and the increase in the fluorescence intensity can be visualized under UV light as well. The following substrates can be used for each enzyme activity:

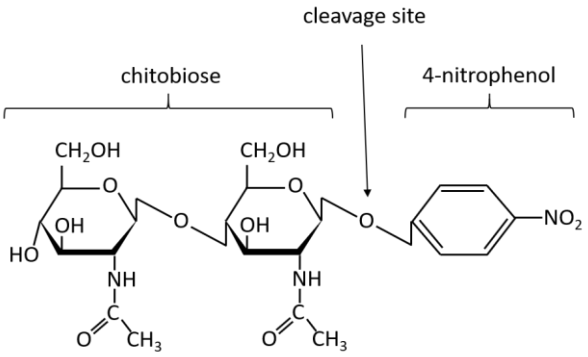
i) Endochitinase: 4-Nitrophenyl β-D-N,N',N''-triacetylchitotriose (chomogenic)
4-Methylumbelliferyl β-D-N,N',N''-triacetylchitotrioside (fluorogenic)

Background of the 4-Nitrophenyl β-D-N,N',N''-triacetylchitotriose hydrolysis:



ii) Chitobiosidase: 4-Nitrophenyl N,N'-diacetyl-β-D-chitobioside (chomogenic)
4-Methylumbelliferyl-N,N'-diacetyl-β-D-chitobioside (fluorogenic)

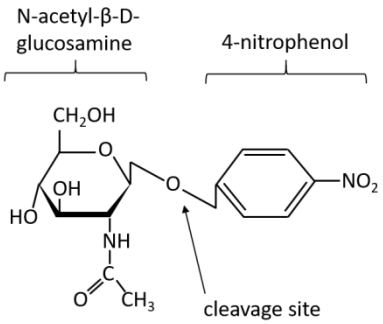
Background of the 4-Nitrophenyl N,N'-diacetyl-β-D-chitobioside hydrolysis:



iii) N-acetyl glucosaminidase: 4-nitrophenyl N-acetyl-β-D-glucosaminide (chomogenic)

4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (fluorogenic)

Background of the 4-nitrophenyl N-acetyl-β-D-glucosaminide hydrolysis:



3.3.2.1. Detection of extracellular chitinase activity - Practice 28 -

Goal

To investigate the chitinase production of *Bacillus*, *Pseudomonas* and *Trichoderma* isolates in plate tests using colloidal chitin as substrate.

Required materials

Inductive medium for bacteria, inductive medium for fungi, 10% colloidal chitin stock solution, 8 Petri dishes, inoculation loop, 1000-5000 µl pipette, Amido Black staining solution, 50 µg/ml 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide stock solution

Inductive medium for bacteria: 0.2% yeast extract, 1% colloidal chitin, 2% agar

Inductive medium for fungi: 1% mannitol, 1% colloidal chitin, 0.5% KH_2PO_4, 0.2% $NaNO_3$, 0.1% $MgSO_4$, 5 µg/ml digitonin, 1.7% agar,

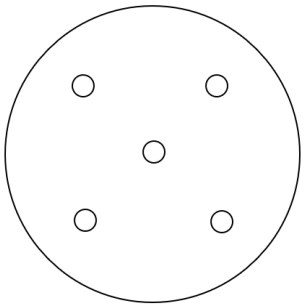
Microorganisms: 5 *Bacillus* isolated from soil during Practice 20 and maintained on T1 agar slants, 5 *Pseudomonas* isolated from soil during Practice 20 and maintained on T1 agar slants, *Trichoderma harzianum* T66, *Trichoderma viride* T114, *Trichoderma atroviride* T122, *Trichoderma viride* T228, *Trichoderma harzianum* T334, *Trichoderma harzianum* T415

Tasks

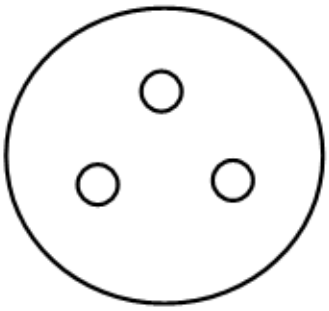
Prepare 100-100 ml of inductive media for bacteria and fungi. After autoclave sterilization, pour the prepared medium into 4-4 Petri dishes. After the medium has solidified, inoculate a corresponding dish with 5 *Bacillus* or 5 *Pseudomonas* (5 isolates/1 Petri dish) or 3-3 *Trichoderma* strains (3 isolates/1 Petri dish). Use inoculation loop. Inoculate one isolate to two different plate because two kinds of detection will be performed.

Follow the layout below to inoculate the bacteria to be tested:

Bacteria:



Fungi:



Then, incubate the dishes at room temperature for a week.

Evaluation

1) Detection with Amido Black solution:

After the incubation period, pipette 5 ml of Amido Black solution onto the surface of four Petri dishes that contained 5 *Bacillus* or 5 *Pseudomonas* or 3-3 *Trichoderma* isolates. Measure the width of the colloidal chitin degradation halo around the colonies. Fill the tables below with the data in millimeters:

Isolates	**Width of colloidal chitin degradation halo (mm)**
Bacillus 1	
Bacillus 2	
Bacillus 3	
Bacillus 4	
Bacillus 5	
Pseudomonas 1	
Pseudomonas 2	
Pseudomonas 3	
Pseudomonas 4	
Pseudomonas 5	

Isolates	**Width of colloidal chitin degradation halo (mm)**
Trichoderma harzianum T66	
Trichoderma viride T114	
Trichoderma atroviride T122	
Trichoderma viride T228	
Trichoderma harzianum T334	
Trichoderma harzianum T415	

2) Detection with fluorogenic reagent:

Measure 5-5 ml of 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide stock solution onto the surface of four Petri dishes that contained 5 *Bacillus* or 5 *Pseudomonas* or 3-3 *Trichoderma* isolates. Incubate the plates at room temperature for 20 min and detect the fluorescence under UV light.

The fluorescence intensity around the colonies is proportional to the chitinase activity of the corresponding isolate. Write down what you have experienced.

3.3.2.2. Measurement of chitinase activity - Practice 29 -

Goal

To investigate and compare of the chitinolytic activity of *Trichoderma* strains cultivated under submerged condition.

Required materials

150 ml inductive broth, 7 100-ml Erlenmeyer flasks, measuring cylinder, 14 Eppendorf tubes, 20-200 µl pipette, 100-1000 µl pipette, centrifuge, 96-well microtiter plate, spectrophotometer (with microplate reader function), 4 mg/ml (11 mM) *p*-nitrophenyl N-acetyl-β-D-glucosaminide stock solution in dimethyl sulfoxide, 100 mM sodium phosphate buffer (pH = 6.8), 10% sodium carbonate solution

Inductive broth: 1% mannitol, 0.5% colloidal chitin, 0.5% KH_2PO_4, 0.2% $NaNO_3$, 0.1% $MgSO_4$
Sodium phosphate buffer (100 mM, pH = 6.8): 49% 100 mM Na_2HPO_4 solution, 51% 100 mM NaH_2PO_4 solution
Sodium carbonate solution (10%): 0.5 g anhydrous sodium carbonate in 5 ml distilled water

Microorganisms: *Trichoderma harzianum* T66, *Trichoderma viride* T114, *Trichoderma atroviride* T122, *Trichoderma viride* T228, *Trichoderma harzianum* T334, *Trichoderma harzianum* T415

Tasks

Inoculation:
Measure 20 ml inductive broth per Erlenmeyer flask and inoculate them with 10 µl from *Trichoderma* suspension; 1 strain/1 flask. Leave one flask as background control (microorganism-free medium). Incubate the flasks for a week at 30 °C.

Sample preparation:

After the incubation, pipette 1-1 ml from each ferment into Eppendorf tubes. Centrifuge them at 10.000 rpm for 5 minutes and transfer the supernatant to a new Eppendorf tube. This clear supernatant will be used as enzyme source for the activity measurements.

Chitinase activity measurement:

Pipette 500 µl of sodium phosphate buffer to 500 µl *p*-nitrophenyl N-acetyl-β-D-glucosaminide stock solution. Then, transfer the clear supernatant to a well of a 96-well microtiter plate, then, add 50 µl of buffered substrate to start the reaction. After 30 min of incubation at 30 °C, add 100 µl 10% sodium carbonate solution to each reaction mixture and monitor the *p*-nitrophenol release (yellow color) at 405 nm using microplate reader. Sodium carbonate stops the reaction immediately and shift the pH to alkaline condition strengthening the intensity of the yellow color change.

Evaluation

Visualize the exochitinase activity of the tested *Trichoderma* strains using bar graph.

Calculation: OD corresponds to enzyme activity = OD of the sample - OD of the background control.

Check your progress

After the completion of the practical courses, answer the following questions and perform the tasks indicated below:

- **Describe the structure of the chitin polymer.**
- **What enzymes can degrade the chitin polymer?**
- **Characterize the chitinolytic enzyme system. List the enzymes involved in the system.**
- **How do citinase enzymes work? Which intermediate and final products are produced during the reactions?**
- **How was the chitinase detection during the practice? Describe the method shortly.**
- **What chromogenic and fluorogenic substrates can be applied to measure chitinolytic activities?**
- **How did we measure the exochitinase activity of fungal strains during the practice? Describe the used substrate and the method shortly.**
- **What is the principle of the chitinase-catalyzed *p*-nitrophenyl N-acetyl β-D-glucosaminide hydrolysis?**

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