Fungal Biotechnology
Laboratory Manual

Prof. Ismail Saadoun
PRACTICAL FUNGI BIOTECHNOLOGY
LABORATORY WORK MANUAL

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PREFACE

This manual has been designed for an undergraduate level laboratory sessions in fungal biotechnology. The manual is divided into experiments that belong to a particular category. An experiment will be carried out each week and some times may be continued in the week after. Prior to each lab session, careful orders and preparations are required which can be found in the procedure or the appendix sections. Each experiment contains the following basic sections:

Introduction
Background and principles behind the assays performed.

Procedure
A detailed description of the materials, equipment needed to conduct the experiment and the method to be followed.

Results
The experimental analysis data are lay out as tables and figures. Reports of the field visits are also included as instructed.

References and further readings
A listing of useful articles and books is also provided.

Appendix
Media, buffers and solutions used in each experiment are provided. Their composition and companies which supply them are also included.

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Contents

<table>
<thead>
<tr>
<th>Topic</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction and Orientation</td>
<td>-</td>
</tr>
<tr>
<td>Fungi: Molds and Yeasts, Revisit</td>
<td>6-10</td>
</tr>
<tr>
<td>Fungi, Revisit</td>
<td>11-13</td>
</tr>
<tr>
<td>Review of Microbial Techniques</td>
<td>14-20</td>
</tr>
<tr>
<td>Mycological Culture Media</td>
<td>21-22</td>
</tr>
<tr>
<td>Induction of Sporulation in Fungal Cultures</td>
<td>23</td>
</tr>
<tr>
<td>Culturing of Filamentous Fungi in Liquid Media</td>
<td>24-25</td>
</tr>
<tr>
<td>Culturing of Yeast-Like Fungi in Liquid Media</td>
<td>26</td>
</tr>
<tr>
<td>Isolation of Fungal DNA, (I. SDS Procedure)</td>
<td>27-29</td>
</tr>
<tr>
<td>DNA Isolation (II. Procedure)</td>
<td>30</td>
</tr>
<tr>
<td>DNA Isolation (III. Procedure)</td>
<td>31-32</td>
</tr>
<tr>
<td>Quantification of DNA</td>
<td>33-34</td>
</tr>
<tr>
<td>Agarose Gel Electrophoresis</td>
<td>35-37</td>
</tr>
<tr>
<td>Appendices</td>
<td>38-40</td>
</tr>
</tbody>
</table>
Lab Schedule

The aim of this lab course is to revisit the fungal structure, characteristics and subdivisions. The focus will be on culturing of filamentous fungi on/in solid and liquid media. Induction of sporulation and DNA isolation from these organisms will be emphasized.

<table>
<thead>
<tr>
<th>Week</th>
<th>Exercise</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Introduction and Orientation</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Fungi: Molds and Yeasts, Revisit</td>
<td>6-10</td>
</tr>
<tr>
<td>3</td>
<td>Fungi, Revisit</td>
<td>11-13</td>
</tr>
<tr>
<td>4</td>
<td>Review of Microbial Techniques</td>
<td>14-20</td>
</tr>
<tr>
<td>5</td>
<td>Mycological Culture Media</td>
<td>21-22</td>
</tr>
<tr>
<td>6</td>
<td>Induction of Sporulation in Fungal Cultures</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Culturing of Filamentous Fungi in Liquid Media</td>
<td>24-25</td>
</tr>
<tr>
<td>8</td>
<td>Mid Term Exam</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Culturing of Yeast-Like Fungi in Liquid Media</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td>Isolation of Fungal DNA, (I. SDS Procedure)</td>
<td>27-29</td>
</tr>
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<td>DNA Isolation (II. Procedure)</td>
<td>30</td>
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<td>13</td>
<td>DNA Isolation (III. Procedure)</td>
<td>31-32</td>
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<tr>
<td>14</td>
<td>Quantification of DNA</td>
<td>33-34</td>
</tr>
<tr>
<td>15</td>
<td>Agarose Gel Electrophoresis</td>
<td>35-37</td>
</tr>
<tr>
<td>16</td>
<td>Final Exam</td>
<td>-</td>
</tr>
</tbody>
</table>
The fungi comprise a large group of eukaryotic nonphotosynthetic organisms that include such diverse forms as the slime molds, water molds, mushrooms, puffballs, bracket fungi, yeasts, and molds. As noted on page 25 the fungi belong to Kingdom Mycetem. The study of fungi is called mycology.

The Mycetem consists of three divisions: Gymnomycota (slime molds), Mastigomycota (water molds and others), and Amastigomycota (yeasts, molds, bracket fungi, etc.). It is the last division that we will study here.

The fungi may be saprophytic or parasitic, and unicellular or filamentous. Some, such as the slime molds, are borderline between fungi and protozoa in that amoeboid characteristics are present and fungilike spores are produced.

The distinguishing characteristics of the group as a whole are that they (1) are eukaryotic, (2) are nonphotosynthetic, (3) lack tissue differentiation, (4) have cell walls of chitin or other polysaccharides, and (5) propagate by spores (sexual and asexual).

In this study we will examine prepared stained slides and slides made from living cultures of yeasts and molds. Molds that are normally present in the air will be cultured and studied macroscopically and microscopically. In addition, an attempt also will be made to identify the various types grown.

Before attempting to identify the various types, familiarize yourself with the basic differences between molds and yeasts. Note in figure 6.1 that the yeasts are essentially unicellular and the molds are multicellular.

Molds Characteristics

Molds are differentiated from each other on the basis of hyphal structure and types of spores present.

Hyphae The individual filaments of molds are called hyphae (hypha, singular). If the filament has crosswalls, it is referred to as being a septate hypha. If no crosswalls are present, the filament is said to be nonseptate, or aseptate. Actually, most of the fungi that are classified as being septate are incompletely septate since the septae have central openings that allow the streaming of cytoplasm from one compartment to the next. A mass of intermeshed hyphae, as seen macroscopically, is a mycelium.

Asexual Spores Molds reproduce by producing spores by both asexual and sexual means. The two principal types of asexual spores are sporangiospores and conidia. Sporangiospores are spores that form within a sac, or sporangium. The sporangia are attached to stalks called sporangiophores. The hyphae are always nonseptate. Rhizopus, Mucor, and Syncephalastrum are representative genera.

![Diagram of mold and yeast structure](image)

**Figure 6.1** Comparison of mold and yeast structure.
nuclear material from the hyphae of two different strains. These fungi also produce sporangiospores.

Subdivision Ascomycotina

All fungi in this subdivision are grouped in one class, the Ascomycetes; consequently, they are commonly referred to as the ascomycetes. They are also called sac fungi. All of them have septate hyphae; most have chitinous walls.

The characteristic sexual ascospores of this class are produced in oval sacs called asci (ascus, singular). Those fungi that produce a single ascus are the ascomycetes yeasts. Other ascomycetes that produce numerous asci in complex fruiting bodies include such organisms as Penicillium and powdery mildews.

Subdivision Basidiomycotina

All fungi in this subdivision belong to one class, the Basidiomycetes. Puffballs, mushrooms, smuts, rusts, and shelf fungi on dead tree branches are also basidiomycetes. The sexual spores of this class are basidiospores, which are produced on club-shaped bodies called basidia. A basidium is considered by some to be a modified type of ascus.

Subdivision Deuteromycotina

There is only one class in this group, the Deuteromycetes. Members of this class are commonly referred to as the fungi imperfecti or deuteromycetes.

This subdivision is, in reality, an artificial group created to include all the fungi that lack sexual means of reproduction. It is a large group, containing over 15,000 species. Whenever it is discovered that a member in this group actually does have a sexual means of reproduction, it is moved up into one of the other subdivisions.

Several options are provided here for the study of molds and yeasts. The procedures to be followed will be outlined by your instructor.

Yeast Study

The organism Saccharomyces cerevisiae, which is used in bread making and alcohol fermentation, will be used for this study. Either prepared slides or living organisms may be used.

Materials:

- prepared slides of Saccharomyces cerevisiae
- broth cultures of Saccharomyces cerevisiae
- microscope slides and cover glasses

Prepared Slides If prepared slides are used, they may be examined under high-dry or oil immersion. One should look for typical blastospores and ascospores. Space is provided on the Laboratory Report for drawing the organisms.

Living Material If broth cultures of Saccharomyces cerevisiae are available they should be examined on a wet mount slide under a phase-contrast or brightfield microscope. Two or three loopsfuls of the organisms should be placed on the slide with an inoculating loop. Oil immersion will reveal the greatest amount of detail. Look for the nucleus and vacuole. They may be difficult to see. The nucleus is the smaller body. Draw a few cells on the Laboratory Report.

Mold Study

Examine a petri plate of Sabouraud's agar that has been exposed to the air for about one hour and incubated at room temperature for 3–5 days. This medium has a low pH, which makes it selective for

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![Types of sexual spores seen in the Amastigomycota.](image-url)
Figure 6.4 Colony characteristics of some of the more common molds.
molds. A good plate will have many different colored colonies. Note the characteristic “cottony” nature of the colonies. Also, look at the bottom of the plate and observe how the colonies differ in color here. The identification of molds is based on surface color, backside color, hyphal structure, and types of spores. Figure 6.4 reveals how some of the more common molds appear when grown on Sabouraud’s agar. Keep in mind in using figure 6.4 that the appearance of a mold colony can change appreciably as it gets older. The photographs in figure 6.4 are of colonies that are 10 to 21 days old.

Conclusive identification cannot be made unless a microscope slide is made to determine the type of hyphae and spores that are present. Figure 6.5 reveals, diagrammatically, the microscopic differences that one looks for in identifying various genera.

**Two Options** In making slides from mold colonies one can make wet mounts directly from the colonies by the procedure outlined here, or make cultured slides as outlined in Exercise 20. The following steps should be used for making stained slides directly from the colonies. Your instructor will indicate the number of identifications that are to be made.

**Materials:**
- mold cultures on Sabouraud’s agar
- microscopic slides and cover glasses
- lactophenol cotton blue stain
- sharp pointed scalpels or dissecting needles

1. Place the uncovered plate on the stage of your microscope and examine the edge of a colored colony with the low-power objective. Look for hyphal structure and spore arrangement. Ignore the white colonies since they generally lack spores and are difficult to identify.

2. Consult figures 6.4 and 6.5 to make a preliminary identification based on colony characteristics and low-power magnification of hyphae and spores.

3. Make a wet mount slide by transferring a small amount of the culture with a sharp scalpel or dissecting needle to a drop of lactophenol cotton blue stain on a slide. Cover with a cover glass and examine under low-power and high-dry objectives. Refer again to figure 6.5 to confirm any conclusions drawn from your previous examination of the edge of the colony.

4. Repeat the above procedure for each different colony.

**Laboratory Report**

After recording your results on the Laboratory Report, answer all the questions.

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**Figure 6.5 Legend**

<table>
<thead>
<tr>
<th>Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Penicillium</em>—bluish-green; “brush” arrangement of phialospores.</td>
</tr>
<tr>
<td>2.</td>
<td><em>Aspergillus</em>—bluish-green with sulfur yellow areas on the surface.</td>
</tr>
<tr>
<td>5.</td>
<td><em>Gliocladium</em>—dark green; conidia (phialospores) borne on phialides, similar to <em>Penicillium</em>; grows faster than <em>Penicillium</em>.</td>
</tr>
<tr>
<td>6.</td>
<td><em>Cladosporium</em> (Hormodendrum)—light green to grayish surface; gray to black back surface; blastoconidia.</td>
</tr>
<tr>
<td>7.</td>
<td><em>Pleospora</em>—tan to green surface with brown to black back; ascospores shown.</td>
</tr>
<tr>
<td>8.</td>
<td><em>Scopulariopsis</em>—light brown; rough walled microconidia.</td>
</tr>
<tr>
<td>10.</td>
<td><em>Alternaria</em>—black surface with gray periphery; black on reverse side; chains of macroconidia.</td>
</tr>
<tr>
<td>11.</td>
<td><em>Helminthosporium</em>—black surface with grayish periphery; macroconidia shown.</td>
</tr>
<tr>
<td>12.</td>
<td><em>Pulularia</em>—black, shiny, leathery surface; thick-walled, budding spores.</td>
</tr>
<tr>
<td>13.</td>
<td><em>Diaposporum</em>—buff-colored wooly surface, reverse side has red center surrounded by brown.</td>
</tr>
<tr>
<td>14.</td>
<td><em>Oospora</em> (Geotrichum)—buff-colored surface; hyphae break up into thin-walled rectangular arthrospores.</td>
</tr>
<tr>
<td>15.</td>
<td><em>Fusarium</em>—variants of yellow, orange, red, and purple colonies; sickle-shaped macroconidia.</td>
</tr>
<tr>
<td>16.</td>
<td><em>Trichotheceum</em>—white to pink surface; two-celled conidia.</td>
</tr>
<tr>
<td>17.</td>
<td><em>Mucor</em>—white to dark gray mycelium; nonseptate; sporangia with sporangiospores.</td>
</tr>
<tr>
<td>18.</td>
<td><em>Rhizopus</em>—white to dark gray; nonseptate; rootlike mycelia; sporangiospores.</td>
</tr>
<tr>
<td>19.</td>
<td><em>Syncephalastrum</em>—white to dark gray surface; nonseptate; sporangiospores.</td>
</tr>
<tr>
<td>20.</td>
<td><em>Nigrospora</em>—white to gray surface; reverse side is black.</td>
</tr>
</tbody>
</table>
Figure 6.5  Microscopic appearance of some of the more common molds (refer to legend on opposite page).
Fungi, Revisit

**Fungi**: a very large heterogenous group of eukaryotic organisms made up of mass of hyphae called mycelium that modified in adverse conditions to resist temp, pH and desiccation

**Habitat**: mainly in terrestrial habitat (soil), fresh and marine water

**Importance**:
1. Harmful
   a. plant diseases, animal and human diseases
   b. spoilage of food and any material that are subject to fungal attack

2. Beneficial:
   a. Degraders: complex OM → simple OM
   b. Industry: fermentation (bread, wine, beer), organic acids, cheese, antibiotics, pr→SCP→Animal food

**Structure**:
Many mycologists thought that plants originate from fungi or fungi identified as plants because
1. their complex C.W that made up of chitin, cellulose or glycogen that give it fairly stable shape
2. immobile habit: however, 200 spores are actively mobile
3. Absorption of food through enzyme excretion

In spite of these similarities, they are not photosynthetic and this is a big difference.

**Body of fungi**:
1. permanent C.W: most fungi have chitin in the C.W
2. Filamentous like structure called mycelium (Greek=Fungus) and mycelium consists of hyphae (Greek=Web) of 10-50 µm in diameter

Hyphae is cylinder in structure, simple morphology can be linear or branched. Can be open tubules (non-septated) or can be septated by transverse septa. With a single or multiple pores

Many fungi, especially the pathogenic one undergo what called YM shift:
Yeast (y) in animal → mold or mycelium (M) form in response to env. Change (Temp, nutrients)

In plant →

Yeast (y) in animal → mold or mycelium (M) form in response to env. Change (Temp, nutrients)

These called Dimorphic fungi:
- e.g. *Candida albicans*, *Histoplasma Capsulatum* (pathogenic)
Nutrition and Metabolism
- most of them are Saprophytes (living on dead organic material) chemoheterotrophs
- Optimum pH for growth = 5.5
- Optimum Temp. for growth = 20-35°C
- Store glycogen in
- Usually aerobic, some yeasts are fac. Anaerobes, no oblig. Anaerobes

Reproduction:
A- a sexual : no union of sex cells (nuclei) or sex organs
1- Transverse fission

2- Hyphal fragmentation resulting in arthrospores, cell fragment behaves as spore

3- Hyphal fragmentation in chlamydospores

4- Sporangiospores in a sporangium

5- Conidiospores arranged in chains at the end of a conidiophore

6- Spores produces from a vegetative mother cell by budding are called Blastospores

B- sexual: union of nuclei
1- fusion between haploid gametes, diploid Zygote 2N

\[
\text{Dikaryotic Stage} \rightarrow \text{Parent cell} \rightarrow \text{Develop} \rightarrow \text{2-haploid spore + haploid spore} \rightarrow \text{Zygote 2N} \rightarrow \text{Zygospore 2N}
\]

Classification
Division Zygomycto  Zygomycetes
Division Ascomycota  Sac fungi (Ascomycetes)
Division Basidiomycota  Club fungi (Basidiomycetes)
Division Deuteromycota  Fungi Impefecti

1-Division Zygomycto
- most of them live on decaying plants and animals in soil and few are parasites on plant, animals and human
- hyplae is not Septated (Coenocytic)
reproduce by -Asexual : Sporangiospores
- Sexual : resulted in Zygospores formation

Ex: Bread mold (Rhizopus stolonifer)
usually reproduce asexually

Application:
food : cheese, meat tenderizers
Industry : alcohol
Pharmacy and medicine: birth control, anesthetics
2-Division Ascomycota:
- Food spoilage
- Plant pathogens
- Genetic and biochemical studies: *Neurospora crassa*
- Septated hyphae

- Asexual reproduction: produce conidiospores (most of them) (multinucleate)
- Sexual reproduction: produce ascus with 1 N ascospores inside

Ex: Yeasts → 40 genera → 350 species. → Reproduce mostly asexually

3-Division Basidiomycota:
Smuts, rusts: Damage cereal crops
Mushrooms and birds nest fungi
Ex: *Agaricus campestris* → Food (Mushroom)
*Amanita* → Poisonous
*Cryptococcus neoformans*: Human pathogen → Cryptococcosis (CNS and lung infection)

4-Division Deuteromycota:
- Lack sexual phase (perfect stage) of reproduction → called imperfect fungi
- Mostly terrestrial (saprophytes, parasites on plants few on Nematodes, human pathogens (athlete’s foot, ringworm and histoplasmosis) industry: *Penicillium* → penicillin

Ex: *Aspergillus* → Organic acids, secondary metabolites (aflatoxine), trichotheccenes: pr inhibitor

Slime Molds and water Molds
Division Myxomycota
Division Acrasiomycota
Division Oomycota
Review of Microbial Techniques

CULTURAL TRANSFER

The procedure for transferring a microbial sample from a broth or solid medium or from a food sample is basically the same. The sample is collected with a sterile utensil and transferred aseptically to a sterile vessel. Two implements commonly used for collecting and transferring inoculum are the cotton swab and the platinum needle or loop. The swab is used in instances where its soft nature and its fibrous qualities are desired such as in taking a throat mucus sample or in sampling the skin of an apple. A platinum needle or loop is used in those instances where a more concentrated microbial sample is available, such as in a contaminated water sample. A typical culture transfer proceeds as follows:

1. In one hand hold the wire loop as you would a pencil;
2. Heat the wire loop until red;
3. Allow it to cool for a moment (this prevents burning or boiling of the medium when it contacts the loop);
4. Holding the culture container in the other hand, remove the cover by grasping it between the small finger and the palm of the loop holding hand;
5. Flame the container by passing the vessel top through the flame slowly (2 to 3 sec) in order to sterilize the rim;
6. Insert the wire loop and take the sample;
7. Re-flame the container top and replace the lid;
8. Open and flame the top of the receiving vessel as you did with the sample vessel;
9. Inoculate the sample into the vessel;
10. Re-flame and cap the receiving vessel;
11. Flame the loop to resterilize it.

All vessels used need to be clearly labeled for identification. The date and name of the person using the vessel should be included along with the other pertinent information, (e.g., medium type, control, concentration, etc.). All swabs, medium tubes, culture plates, and other items contaminated with microbes should be autoclaved before washing or disposal.

PLATING

Isolation of individual microbial types may be obtained by dilution methods. The dilution, a reduction of microbial cell concentration, may be achieved by spreading a small amount of culture across a wide medium surface. This technique is called streaking. Bacterial cell dilution may also be carried out using a series dilution scheme, a small amount of initially concentrated culture is introduced into a volume of medium or physiological saline and then homogeneously dispersed into that volume. Physiological saline (0.85% NaCl) is used to protect cells from sudden osmotic shock thus preventing cell rupture, a sample of the new volume may be redispersed in yet another dilution volume to achieve further cell number reduction, by transferring known volumes of sample culture to known volumes of dilution media, one can calculate the reduction in cell concentration achieved, for example, if one introduced 1 ml of a sample into 9 ml of medium, one would have reduced the initial concentration by a factor of ten.

Please refer to a dilution scheme for practice in making dilutions. In dilution schemes one must maintain aseptic technique. All transferring items must be microbe free. All new media or dilution media must be sterile.
A pipet is used to transfer volumes of liquid. The pipette should be clean and sterile. It should be equipped with a pipette bulb or pro-pipette so that oral contact and the potential danger of inhaling the microbial sample is avoided. Always place pipettes in germicidal washing solution immediately after use.

Dilution of cultures made by volume dilution may be plated out in Petri dishes and then incubated to allow the microbes time to grow. A typical plating procedure would be as follows:

1. Pipette 1 or 0.1 ml of a known dilution of a sample into the bottom section (smaller plate) of a sterile Petri dish;

2. Within 20 min add 12-15 ml of warm (46-48°C) fluid medium to this Petri dish;

3. Cover the dish;

4. Swirl it gently to disperse the sample throughout the medium, (a figure eight pattern holding the dish flat on the table is the recommended swirl pattern: care should be taken to prevent splashing of the medium onto the lid of the dish);

5. Allow the plate to stand, cool, and solidify;

6. Invert the Petri dish (medium surface pointing down) and incubate in this position.

Petri dishes are incubated upside down to prevent water from condensation from standing on the medium surface during incubation. Pools of surface water would result in the loss of individual surface colonies since bacterial cells forming in the colonies could use the water pools as vehicles to reach the medium. After a period of incubation microbial growth may be observed. If sufficient dilution has been achieved, individual colonies of microbes may be clearly seen. It is assumed that colonies arises from single microbial cells, thus an individual colony represents only one microbial type. This assumes that the microbes in the original culture were not clustered and that a true homogenous dispersion was achieved. (Shaking the solution with glass beads helps to break up cells clusters.) by picking out individual colonies and transferring them to a new sterile medium, microbial isolation can be achieved.

Isolation is also achieved using the streaking technique. This involves the aseptic transfer of a small quantity of culture to a sterile Petri dish containing medium. The most common implement for streaking is the wire loop.

Streaks should be performed by initially introducing an inoculum of the culture onto a small area of the medium plate surface. This is called ‘the well’. After inoculating the well, the transfer loop is re-flamed, allowed to cool, and then touched on a remote corner of the plate to remove any heat remaining. Beginning with the sterile loop in the well a streak is made across a corner of the medium surface. (This spreads a bit of the culture out over the medium—dispersing or diluting the culture.) the loop is re-flamed, cooled, and the streaking continued until all the available medium surface is utilized. On a typical plate 3-5 streaks can be made.
Remember: the streaking loop must be re-flamed after each streak.

Both processes, streaking and volume dilution reduce and disperse the cell concentration onto the medium. Upon incubation both dilution procedures should produce isolated colonies of a single strain. The dilution technique has added use, in that upon sufficient dilution, all the colonies from the dilution can be seen as separate individual spots when plated. By counting these spots and multiplying that number by the dilution factor for the plate, one can arrive at an estimate of the number of organisms in the original culture solution.

As a rule of thumb only those incubated plates which have between 30 and 300 colonies are used to determine organism concentration in the original culture. Thirty is taken as the lower limit since statistically this many individual colonies are required for accuracy in calculation. Three hundred is taken to be the upper limit because difficulty is encountered in counting more than this number of colonies accurately.

Motility Testing
Many microbes are motile. Motility can be checked by inoculating a culture sample into a semisolid medium. This is done with an inoculating needle which is stabbed straight down and pulled straight out of the tube. Upon incubation, a non-motile colony will produce a single line of growth along the needle jab line, while a motile colony will give a wider band of growth.

The hanging drop mount is used to check motility. It is prepared by placing a ring of lubricating grease around the rim of the recession in the hanging drop slide. A drop of culture medium or a water suspension of a culture is then placed on a cover slip. The cover slip is inverted so that the drop is clinging to the lower side, and the cover slip is laid to rest on the slide—being supported by the ring of grease. This mount has the advantages that motility of live, motile microbes can be observed.

Staining
A method of biochemical differentiation is staining. Staining operates on the principal that different types of microbes have different chemical constituents making up their cellular components. For example, the Gram stain operates on the principle that some cells retain a crystal violet-iodine complex after leaching with an alcohol solvent, these cells generally have complex membranes which result in retention of the blue complex and are thus called gram positive. Other microbials with less complex membranes are not affected by the mordant, iodine. The dye in these cells is washed out and replaced by a safranin counter-stain (red).
These cells are said to be gram negative. There are many other types of cellular dyes. There are basic dyes specific for nuclear material, other cellular elements, and spores.

**Objectives:**

This exercise will review the technical skills required to successfully function in an analytical microbiology laboratory. This exercise will enable you to:

1. Transfer cultures, streak plates and inoculate slants;
2. Carry out dilution schemes to obtain microbial counts;
3. Determine microbial motility by two methods;
4. Carry out gram and spore stains;

**Materials:**

Broth and slant of: *Escherichia coli, Bacillus subtilis, Staphylococcus aureus*

Broth mix of: *Staphylococcus aureus* and *Escherichia coli*

Tryptone glucose extract agar (TGEA)

1 ml pipettes

Petri plates

99 ml dilution blanks

Gram and spore stains

Semi-solid agar tubes

**Procedure:**

**A. Microbial Isolation**

1. Flask of agar medium are kept in a 48°C oven to maintain their fluidity, label ____
   plates of TGEA and pour 1—15 ml of the medium into these plates and allow them to cool and solidify for streaking and spread plating.
2. The instructors have prepared 4 different types of broth cultures. You will dilute out each of these 4 different cultures, 2 by spread plating techniques and 2 by pour plating methods. Your instructor will explain these procedures, as well as designate which of the cultures are to be spread or pour plated and to what dilution. Dilution schemes should be worked out first on paper to avoid confusion. (Note —examples of dilution schemes are given at the end of this exercise)
3. If the TGEA plates prepared in step 1 have solidified, proceed to streaking so that isolated colonies may be observed. Streak out samples from all 4 broth cultures.

   Which of the cultures are to be spread or pour plated and to what dilution? Dilution schemes should be worked out first on paper to avoid confusion. (Note —examples of dilution schemes are given at the end of this exercise)
4. When all plates have cooled and solidified, invert and incubate at 37 c for 48 hr. Count the plates from the dilution(s) yielding between 25 and 250 colonies. Calculate the
bacterial cell concentration in the original culture. Observe the streak plates. Exchange class data.

**B. Microbial Motility**

1. Obtain 3 tubes of semi-solid agar and inoculate each tube with one of the 3 culture types using an inoculating needle. Omit the mixed culture sure to label each tube, incubate tubes at 37°C for 48 hr.

**C. Staining**

Use the broth cultures provided and the plates streaked for isolation as sources for microorganisms to stain.

1. Make gram stains of the *E. coli, S. aureus, B. subtilis* and the mixed culture according to the procedure described by your instructor. Observe these stains under the microscope using the oil immersion magnification.

2. Make a spore stain of the cultures assigned to you. Observe it under the microscope using the oil immersion objective. Can you observe distinct spore bodies? If so, are they terminal, subterminal, or central? Are cells swollen at the spore location?

**Dilution Calculations**

Dilution factor = initial dilution x subsequent dilutions x amount plated

Count per ml (or g) = reciprocal of dilution factor x colonies counted

**Example:**

A sample was diluted initially 1:100 (1 ml of in 99 ml sterile diluent). A subsequent 1:10 dilution (1 ml of the initial dilution into 9 ml sterile water) was prepared. Finally, 0.2 ml of the final dilution plated and 64 colonies were counted on the plate.

Initial dilution x subsequent dilutions x amount plated = dilution factor

\[
\frac{1}{100} \times \frac{1}{10} \times 0.2 = 0.0002 \quad \text{or} \quad 10^{-2} \times 10^{-1} \times 2\times 10^{-1} = 2 \times 10^{-4}
\]

reciprocal of dilution factor x colonies counted = count per ml

5000 (or 5 x 10^3) x 64 = 320,000 (or 3.2 x 10^5)

**A.** Plate count results should be reported to two significant figures only.

**Example:**

If the dilution factor used was 10^6 and 212 colonies were counted, the count/ml would be calculated thus,

\[
\text{Reciprocal of dilution factor x colonies counted} = \frac{\text{count/ml}}{10^6}
\]

\[
10^6 \times 212 = 2.12 \times 10^8
\]

Then, the answer should be rounded off to 2.1 x 10^8 colony forming units (CFU) per ml.

**B.** Only those plates with between 25 and 250 colonies should be used to calculate plate counts.
Counting Colonies on Plates and Recording Results

Refer to the prepared handout for details.

References:
Dilution Examples

A. Using 9 ml Dilution Blanks

Original Sample

Liquid (1 ml)  
Solid (1 g)

B. Using 99 ml Dilution Blanks

Original Sample

1 ml or 1 g

C. Combinations

Original Sample
Mycological Culture Media

General Natural Culture Media

These culture media are characterized by being made of and/or one of their components is a natural organic product of non specified constituents. Being general media means it is not made for of specific group or kind of microorganisms; rather it is suitable for more than one group and many kinds of other organisms. One example of such culture media is the Potato Dextrose Agar (PDA).

Preparation of PDA

Components:
- Slices Potato tissue 200g
- Dextrose 20g (This could be replaced by sucrose 10g)
- Agar 20g (This could be reduced according to the desired hardness degree of the solidified media)
- Distilled or Deionized water 1000ml (1000g)

Procedure:

Boil the potato slices inside 500ml of the distilled water until it gets to be soft, i.e. cooked. Strain the cooked potato though several layers of cheese cloth and collect 500ml of Potato Broth. At the same time, dissolve the Agar and Dextrose in another 500ml of distilled water. This is prepared by gradual addition of the agar powder to the heated water inside a large beaker (about 80-90°C) with continued stirring in order to avoid clumping of the agar and burning of the agar on the bottom surface of the beaker. Add the dextrose and continue stirring and maintaining the high temperature (above 70°C) until the entire agar get completely dissolved. At this stage the agar dextrose solution should be homogenous clear medium, i.e. it shows no tiny swelled agar particles. Mix both solutions, the Potato Broth (500ml) and the Agar Dextrose (500ml) to gather making 1000ml of PDA. Now this culture media is ready for sterilization.

This culture media should be distributed into suitable containers (conical flasks) with their mouth plugged with cotton plugs (some may be closed by screw caps which should be left one half turn open, i.e. not tightly closed) before sterilization. Place those containers inside an autoclave (one can use a Pressure cooker) and accomplish complete sterilization under 121°C and 15psi (1.1 Bar of steam pressure) for about 15-20 min depending on amount of media in each container as larger volume require longer time).

Now this PDA has a pH value of about 6-8 (neutral acidity) and it could be poured into Petri dishes once its temperature becomes close to 42°C, but not lower as it starts to solidify and does not pour evenly into the plate.

Though the PDA is a natural and general (non selective) but it could be modified to be rather selective media for supporting growth of fungi rather than bacteria. This could be accomplished by making the reaction of the media acidic using organic acid, the lactic acid. Acidification is done by adding lactic acid (25%) to the sterilized media (not before sterilization) bringing its reaction to pH4. Further selectivity could be achieved by the addition of certain antibiotics (anti-fungal or bacteria). Addition of anti-fugal agent to the prepared PDA may make it more specific for bacterial growth. Selectivity in this media could
also be accomplished by raising its osmotic potential via adding 100g of Na Cl per liter (10%).

PDA culture media are also marketed as ready- made powder which is used according to instruction on the container.

Other Culture Media used for Fungi

MA (Malt Extract Agar): 30 g/l malt extract (Difco Laboratories), 15 g/l agar-agar, pH 7.0
CMA (Cornmeal Agar): cornmeal extract, 15 g/l agar-agar, pH 6.0. Cornmeal extract is prepared as follows: boil 50g of ground maize grain wrapped in a cloth in a water bath. After simmering for 1 h, squeeze the extract through the cloth, and adjust to 1l with sterile water.
CZA (Czapek-Dox agar): Czapek-Dox broth (30g/l glucose, 5g/l yeast extract, 3g/l NaNO3, 1 g/l K2HPO4, 0.5 g/l MgSO4-7H2O, 0.01 g/l FeSO4-7H2O, 15 g/l agar-agar, pH 6.0 to 6.5.
YPD-agar (yeast-peptone-dextrose agar): YPD medium (20 g/l glucose, 10 g/l yeast extract, 20 g/l peptone), 15 g/l agar-agar

Semi-Synthetic and Synthetic Culture Media

This culture media is basically made of salt solution (basic salts) as a source of the necessary nutrient elements for the fungal growth plus a source of carbon (any organic or inorganic carbon containing compounds). The media becomes a synthetic one (for example CZA) once it has all of its chemical constituents chemically defined. Such media is made of the basic salts and glucose as the sole carbon source. Both kinds of culture media can be used as broth (liquid) or solidified with agar.

Selective culture media

This culture media is designed for the growth and /or suppression of certain group or individual microorganism. This selectivity can be accomplished to different degrees according to the constituents of the culture media itself, such as its reaction (pH), osmotic potential (salt concentration), inhibitors and promoters (antibiotics, specific growth promoters).

Usual manner of using the culture media

All kind of mycological culture media are used as broth (no agar added) or solidified by agar. The liquid, or broth culture media is used in conical flasks and bottles with their mouth closed by screw cap or cotton plug. The solidified culture media used as poured in Petri dishes, jars or slant tubes. The two are also their mouth should be closed with screw cap or cotton plugs.

Inoculation

Inoculation is accomplished by introducing a tiny amount of the fungal living cell being either propagation structure (spores, sclerotia, etc)or vegetative cells(single cells or hyphae)

Long-Term Maintenance medium of Fungal Cultures:

1- Storage in sterile Water
2- Storage in Culture Tubes (Slants)
3- Storage as Frozen Stocks
**Induction of Sporulation in Fungal Cultures**

Induction of population is necessary for two purposes: (1) establishment of single spore-derived culture and (2) large-scale production of mycelia from filamentous fungi. Since each spore grows into one mycelium, inoculation of high number of spores into liquid medium produces a large amount of fungal material in a short period of time. A special nitrogen-deficient medium is often used for high accumulation of spores.

**Solutions:**
Nitrogen-deficient 10 g/l potassium acetate, 1 g/l yeast extract, 0.5 g/l glucose
Washing solution: 0.1 M NaCl, 0.5% Triton X-100

**Method:**
1- Pipette 25-ml aliquots of the autoclaved medium into sterile 100-ml Erlenmeyer flasks. Work in a biological safety hood.
2- Lean Erlenmeyer flasks against a support at an angle of about 70 degrees, and allow the agar to solidify.
3- Using a sterile inoculation loop, transfer a single colony from an agar plate to the sporulation flask, and distribute the cells on the agar surface.
4- Incubate for 3 to 4 days at the required temperature generally 23 to 28 degrees; leaving caps loose).
5- To harvest the spores, wash the agar surface with 1 to 2 ml of sterile washing solution.
6- Spore suspensions can be stored at 4 C and should be used within 2 weeks of harvest. The number of spores may be calculated with the help of a counting chamber (e.g. hemocytometer, Thoma chamber) under microscope.
Culturing of Filamentous Fungi in Liquid Media

Media

General fungal culture medium [Glucose- Yeast Extract- Peptone Media (GYPM)];
30 g/l glucose, 2 g/l yeast extract, 2 g/l peptone, mineral salts and trace elements mix stock [10 ml/l A, 10 ml/l B, 10 ml/l C1 ml/l D (see table below)], pH 6.0. Solutions A and B are added before autoclaving the liquid of solid media. Solutions C and D are filter sterilized and added after autoclaving. This rich media is used for the growth of mycelia of, e. g. species of the genera *Chetomium*, *Fusarium*, *Gibberella*, *Mucor*, and *Verticillium*.

CYM-Complete culture medium;
20 g/l dextrose, 2 g/l yeast-extract, 2 g/l peptone, 1 g/l K2HPO4, 0.46 g/l KH2PO4, 0.5 g/l MgSO4-7H2O, pH 7.0. This medium is used for the growth of mycelia of e. g. species of the genera *Absidia*, *Mucor*, and *Morcerella*.

V8-juice Medium;
200 ml/l V8-juice (unfiltered multivitamin juice), 3 g/l CaCO3, pH 7.2. This medium is used for the growth of mycelia of, e. g. species of the genus *Leptosphaeria*.

Malt- Yeast Broth;
3 g/l malt extract, 3 g/l yeast extract, 5 g/l peptone, 10 g/l glucose, pH 7.5. This medium is used for the growth of mycelia of, e. g. species of the genus *Leptosphaeria*.

Czapek-Dox medium;
30 g/l glucose, 5 g/l yeast extract, 3 g/l NaNO3, 1 g/l K2HPO4$, 0.5 g/l MgSO4-7H2O, 0.01 g/l FeSO4-7H2O, pH 6.0 to 6.5. This medium is used for the growth of mycelia of, e. g. species of the genera Trichoderma, *Aspergillus*, and *Emericella*.

Method

1- Transfer 100-ml aliquots of the sterilized medium into 500-ml sterile Erlenmeyer or baffled bottom flasks. Work in a biological safety hood.
2- Inoculate each flask with spore suspension (final spore density $10^8$ /ml). If hyphal inoculums is used, the number of starting hyphae may be increased by per-culturing the mycelia in small liquid culture followed by disruption in a sterile blender.
3- Incubate for 72 h (caps loose) on a rotary shaker (220rpm) at the required temperature (most filamentous fungi grow well at 25 to 28 C; however, some species have other requirements)
4- To harvest the mycelia, filter the culture medium under vacuum though a Buchner funnel or centrifuge it for 15 min (2500 X g, room temperature).
5- After harvest, wash mycelia twice with sterile water. Mycelia may now immediately be used for DNA isolation or stored by freezing in liquid nitrogen. Frozen mycelia may be kept at -20C for days or at -70C for up to 1 year. Alternatively, frozen mycelia may be lyophilized and stored in a dry and dark place for several years at room temperature.
<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Ingredients</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Phosphate buffer</td>
<td>1.5 g/l KH2PO4, 0.2 g/l K2HPO4</td>
</tr>
<tr>
<td>B</td>
<td>Mineral Salt and trace elements solution</td>
<td>2.00 g/l NH4NO3, 0.4 g/l NaSO4, 0.15 g/l MgSO4-7H2O, 0.02 g/l MnSO4-4H2O, 0.02 g/l CuSO4-5H2O, 0.02 g/l ZnSO4-7H2O</td>
</tr>
<tr>
<td>C</td>
<td>Ferric solution</td>
<td>0.15 g/l Fe(NH4)2SO4</td>
</tr>
<tr>
<td>D</td>
<td>Calcium solution</td>
<td>1.10 g/l CaCl2</td>
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</tbody>
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Culturing of Yeast-like Fungi in Liquid Culture Media

Culture Media

Yeast Peptone Dextrose broth culture media (YPD broth):
20 g/l glucose, 10 g/l yeast extract, 20 g/l peptone. This medium is widely used for yeasts (e.g. *Canida, Saccharomyces, Cryptococcus*) if no special growth conditions are required.

Yeast Nitrogen Broth culture media (YNB):
1.7 g/l yeast-nitrogen base without amino acids and ammonium sulfate, 5 g/l (NH4)2SO4, 20 g/l glucose. This minimal medium supports growth of several yeast-like fungal species which have no special nutritional requirements.

Method
1- Transfer 3-ml aliquots of the sterile liquid medium into 10-ml sterile culture tubes. Work is a biological safety hood.
2- Use sterile loop to inoculate each tube with a single yeast colony from a culture plate.
3- Incubate overnight at 30°C in a shaking incubator at 220rpm (some yeast species may require other temperatures; e.g. 37°C is optimal for *Cryptococcus neoformans*).
4- Transfer the 3-ml overnight cultures into 50-ml Erlenmyer flasks containing fresh medium.
5- Incubate for 48h at 30°C in a shaking incubator at 220 rpm.
6- To harvest the yeast cells, centrifuge the culture medium for 15min (2500X g, room temperature).
7- Wash the yeast cells twice with sterile water. The cells may now immediately be used for DNA isolation or stored by freezing in liquid nitrogen. Frozen cells may be kept at -20°C for few days or at -70°C for up to 1 year.
Isolation of Fungal DNA

Sources of fungal material

1- Plant material, Soil and Air-borne Spores
2- Human and Animal Pathogenic Fungi

Large-Scale Accumulation of Fungal Material for DNA Isolation

Fungal material should be grown in liquid rather than solidified media prior to DNA isolation for two reasons. First, the use of liquid media allows the accumulation of large amounts of mycelia or cells in a short period of time (see above). Second, polysaccharides (i.e. agar-agar attached to the fungal mycelia) may render DNA isolation difficult. If culturing on agar plates is necessary for one or the other reason, the agar should be carefully stripped off the mycelia prior to DNA isolation.

Efficient aeration is needed for large-scale growth. To ensure this, the medium should constitute more than one fifth of the total volume of the (preferably Erlenmeyer) flask, and culturing should be performed on a shaking incubator at 220rpm. Any media which offer favorable growing conditions for the investigated fungal species can be used. Some commonly used media are described below. However, some species may require very special media.

Filamentous fungi and yeast have fundamentally different growth characteristics and nutritional requirements. While yeast-like fungi are generally characterized by fast growth rates (i.e., short cell cycles) and modest requirements on their growth media, filamentous fungi usually exhibit slow growth rates (i.e., longer cell cycles) and often have highly specific demands on media composition (e.g., the inclusion of certain trace elements). Procedures for both types of fungi are given below.

In general, the ingredients for liquid media are dissolved in water, aliquoted into Erlenmeyer flasks, and autoclaved. Small amounts may also be filter sterilized, which circumvents the risk of caramelization (e.g., of glucose).
DNA Isolation

There is no consensus on optimal stage of fungal culture for DNA isolation. Some researchers claim that cultures should be derived from the log phase, while others prefer stationary phase. Cell wall disruption is usually easier in the log phase. The efficient disruption of the cell wall is one of the critical steps during DNA isolation. Fungal cell walls contain chitin and often highly resistant to mechanical forces. Several strategies to destroy the cell wall may be followed. One commonly used technique makes use of sand or small glass beads to grind fresh mycelium in a mortar with a pestle. This is reliable and inexpensive, but many hyphal segments remain intact. A more efficient method (also often used for plant tissues), which is more favored, is to freeze the mycelia or cells in liquid nitrogen prior to grinding with mortar and pestle. It is important to keep the powder frozen by adding liquid nitrogen throughout the procedure. Still another technique involves cell wall degrading enzymes to generate protoplasts prior to DNA isolation. This method avoids mechanical force and yield highly intact genomic DNA, but is time consuming and expensive.

A large variety of methods to isolate fungal DNA have been described. However, the majority of methods belong to one of the following categories:

1. SDS- or TNS/PAS-based methods. This strategy makes use of strong detergents (SDS: sodium dodecyl sulfate, TNS/PAS: Triisopropyl-naphthalene sulfonic acid/para-aminosalicylic acid) to break the cell and organelle membranes.
2. CTAB-based methods. In this strategy, the DNA-binding detergent CTAB is included in isolation buffer. CTAB-based methods are also often used for plants.
3. Methods based on protoplast isolation. Here, specific mixture of cell wall-degrading enzymes (e.g. zymolase) is used to prepare protoplasts, from which DNA extraction is easier to perform. This technique is very useful for fungal cells with strong cell walls or a large capsule (such as Cryptococcus neoformans)

The extraction methods described below are successfully used in the laboratory. Only minimum equipment is required, and DNA is usually sufficiently pure for PCR and restriction experiments.

I. SDS Procedure

Three different SDS-based procedures are given here, two of which are minipreps. The first protocol states that; following initial cell wall disruption, the fungal cells are lysed by addition of an SDS-containing isolation buffer. Proteins are removed by protease K treatment. And RNA is eliminated by RNAase.
**Solutions**

Liquid Nitrogen  
Extraction buffer: 25 mM Tris-HCl, 25 mM EDTA, 50 mM NaCl, 1% SDS, pH 8.0  
5M NaCl  
Proteinase K: 20 g/ml  
RNase A: 10 mg/ml (to inactivate contaminating DNases, the RNases has to be preincubated for 15 min at 100°C)  
Phenol (saturated with 1X TE; refer to safety precautions)  
Ratio of chloroform to isooamy alcohol (24:1)  
3M sodium acetate, pH 5.2  
96% Ethanol  
70% Ethanol  
1X TE; 10mM Tris-HCL, 1mM EDTA, pH8.0

**Method**

1. Grind 2g of frozen mycelium in liquid nitrogen using a mortar and pestle. Do not allow the powder to thaw.  
2. Suspend the powder in 25 ml of extraction buffer in a centrifuge tube, and mix gently.  
3. Incubate for 1h at 0°C.  
4. Add 5M NaCl to a final concentration of 1M, mix gently, and incubate for 1h at 0°C.  
5. Centrifuge for 30min 2500 X g, 4°C) to separate the cell wall and cell membrane fragments from the DNA.  
6. Transfer supernatant to a new tube, and incubate for 10min at 65°C.  
7. Add proteinase K (50 µg/ml final concentration), and incubate for 20min at 37°C.  
8. Add an equal volume of phenol, and shake gently for 2min.  
9. Centrifuge for 15min (2500Xg, room temperature) to separate the phases.  
10. Transfer the aqueous phase to a new tube, add a equal volume of phenol and chloroform (1:1), and shake gently for 2min.  
11. Centrifuge as above.  
12. Transfer the aqueous phase to a new tube, add an equal volume of chloroform and isooamy alcohol (24:1), and shake gently for 2min.  
13. Repeat Step 9, and then transfer the aqueous phase to a new tube.  
14. Add RNase A (50µg/ml final concentration), and incubate for 3h at 37°C.  
15. Add an equal volume of phenol, and shake gently for 2min.  
16. Repeat Step 9 to 13.  
17. Transfer the aqueous phase to a new tube (e. g. 30-ml glass centrifuge tube), and precipitate the DNA by adding 0.03 vol of 3M sodium acetate and 2.5 vol of cold 96% ethanol. Mix well, and incubate for 1h or overnight at -20°C.  
18. Centrifuge for 30min (14,000 X g, 4C).  
19. Wash the DNA pellet twice with 70% ethanol, and centrifuge for 10min(14,000 X g, 4C).  
20. Air dry the DNA pellet, dissolve it in ca. 500µl of 1XTE, and store at +4 or at -20C.
II. The second Protocol states that; following cell wall disruption, cells are lysed, extracted with phenol/chloroform, and the DNA is precipitated with isopropanol. The method can be sued for small amounts of mycelia or cells. This makes it an excellent procedure for the preparation of template DNA for PCR experiments.

Solutions:

Liquid Nitrogen
Lysis buffer; 50mM Tris-HCl, pH 7.2, 50mM EDTA, 3% SDS, 1% β-mercaptoethanol
Phenol (saturated with 1 XTE, refer to safety precaution)
Ratio phenol to chloroform to isoamyl alcohol (25: 24: 1)
3M sodium acetate, pH 8.0 (pH 5.2 also works)
100% isopropanol
70% ethanol
1 X TE; 10mM Tris-HCl, 1mM EDA, pH 8.0

Method

1. Grind 0.1 to 3.0 g of mycelia in microfuge tube in liquid nitrogen using a disposal pestle (a conical grinder, exactly fitting the tube and rotated by hand or an electric potter at 200rpm for several minutes).
2. Add 400µl of lysis buffer, and vortex until the mixture becomes homogenous. If the suspension is too viscous, add more lysis buffer (up to 700µl).
3. Incubate for 1h at 65°C.
4. Add an equal volume of phenol, chloroform and isoamyl alcohol (25: 24: 1), and vortex briefly.
5. Spin in microfuge for 15min (or longer, aqueous phase should be clear) at 14,000 X g at room temperature.
6. Transfer the aqueous phase to a new tube, and precipitate the DNA by adding 0.03 vol of 3M sodium acetate and 0.5 vol of isopropanol. Mix gently but thoroughly.
7. Incubate for at least 30min at 4°C.
8. Spin in a microfuge for 15min (14,000 X g, room temperature).
9. Discard the supernatant. Rinse the pellet once with 70% ethanol.
10. Invert the tube, and drain on a paper towel.
11. Air dry the DNA pellet, dissolve it in an appropriate volume of 1 X TE (e.g. 100 to 500 µl), and store at +4 or -20°C.
III. The third protocol

This protocol avoids the use of hazardous chemicals such as phenol. It is suitable for the extraction of very small amounts of tissue cultured directly in a microfuge tube, which makes it especially useful for preparation of template DNA for PCR experiments.

Solutions:

- Extraction buffer: 200mM Tris-HCl, pH 8.5, 250 mM NaCl, 25mM EDTA, 0.5 % SDS
- 3M sodium acetate, pH 5.2
- 100% isopropanol
- 70% ethanol
- 1 X TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Method

1. Grow the fungal culture in 500µl of liquid media I a 1.5-ml microfuge tube according to one of the methods described above.
2. Pellet the mycelia by centrifugation for 5 min at 15,000 X g in a microfuge.
3. Discard the supernatant, wash the pellet with 1 X TE, and centrifuge as above.
4. Discard the supernatant, and add 300µl of extraction buffer.
5. Grind the mycelium for several minutes with a conical grinder; see Step 1 in the previous method.
6. Add 150 µl of 3M sodium acetate, mix briefly, and incubate for about 10min at -20C.
7. Spin in a microfuge for 15 min (15,000 X g, room temperature).
8. Transfer the supernatant to a new 1.5-ml microfuge tube, and precipitate the genomic DNA by adding an equal volume of isopropanol.
9. Incubate for at least 5 min at room temperature.
10. Centrifuge as above.
11. Rinse the pellet in 70% ethanol. Invert the tube, and drain on a paper towel.
12. Air dry the DNA pellet, dissolve it in 50µl of 1 X TE, and store at +4 or -20C.

TNS/PAS Procedure

Ground mycelia are incubated in a hot extraction buffer containing the detergents TNS (triisopropynaphthalene sulfonic acid) and PAS (para-aminosalicylic acid) as well as phenol. After organic extraction with chloroform, the DNA is precipitated with ethanol.

Solutions

- Liquid Nitrogen
- TNS
- PAS (sodium salt X 2H2O)
- Extraction buffer: 1M Tris-HCl, 1.25 M NaCl, 0.25 M EDTA, pH 8.0
- Distilled water
Phenol (saturated with 1X TE; refer to safety precaution)
Chloroform
96% ethanol
79% ethanol
1X TE; 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

Method

1. Dissolve 100mg of TNS and 600mg of PAS in 10 ml of distilled water in a 100-ml Erlenmeyer flask. Add 2.5 ml of extraction buffer and 7.5 ml of phenol. Preheat to 55C.
2. Grind 2g of frozen mycelia in liquid nitrogen using a mortar and pestle. Do not allow the powder to thaw.
3. Add ground mycelia to the preheated extraction solution. Incubate for 2 min at 55C. Shake occasionally.
4. Add 5ml of chloroform, and incubate for 2 min more at 55C. Shake occasionally.
5. Transfer to centrifuge tube and spin for 10 min (3700 X g, room temperature) to separate the phases.
6. Transfer the aqueous phase (upper phase) to a new tube, and add 10ml of phenol and chloroform (1:1), Shake gently for 2 min at room temperature.
7. Centrifuge as above.
8. Transfer the aqueous phase to a new tube, and add 10ml of chloroform. Shake gently for 2 min.
9. Centrifuge as above.
10. Transfer the aqueous phase to a 30ml centrifuge tube, and precipitate the genomic DNA by adding 0.03 vol of 3M sodium acetate and 2.5 vol of 96% ethanol. Mix well, and incubate for 1 h or overnight at -20C.
11. Centrifuge for 30 min (14,000 X g, 40C).
12. Discard supernatant. Wash the DNA pellet twice with 70% ethanol, and centrifuge for 10 min (14,000 X g, 40C).
13. Air dry the DNA pellet, dissolve it in 0.5 to 1ml of 1XTE, and store at +4 or -20C.
Quantification of DNA

Two procedures are most widely used for estimating DNA concentration.

A. One method is based on spectrophotometric measurement of UV absorbance at 260 nm.
B. The second method is based on the UV-induced fluorescence emitted by ethidium bromide-DNA complexes.

Ethidium Bromide Staining

In this procedure, DNA samples are subjected to agarose gel electrophoresis and subsequently stained with ethidium bromide. The dye intercalates into the DNA double helix, and the intensity of fluorescence induced by UV light is proportional to the amount of DNA in the lane. Comparison to a set of standard, e.g., of lambda DNA, gives an estimate of the amount of DNA in an unknown sample.

This method has the following advantages:
1. Allows DNA quantification.
2. Facilitates estimation of the extent of contamination by RNA.
3. Supports the evaluation of DNA quality (i.e., the extent of degradation).

Solutions:

Ethidium bromide: 10 mg/l in water (refer to safety precautions)
0.8% Agarose in electrophoresis buffer
Electrophoresis buffer: TAB, TBE, or TPE (see previous sections)
Gel-loading buffer: 30% glycerol, 1% SDS, 0.25% bromphenol blue
DNA (e.g., phage lambda): different concentrations (e.g., from 0.01 to 0.2 µg/µl) diluted in water or 1X TE.

Methods

1. Mix an appropriate amount of the DNA sample (e.g., 5µl) with 0.2 vol of gel loading buffer. Mix 5µl of each of a series of lambda DNA standards (this covers a range of 50 to 100 ng of DNA if the concentration given above are used) with 0.2 vol of gel-loading buffer.
2. Load samples alongside with standards onto a 0.8% agarose gel.
3. Electrophorese until the bromphenol blue dye front has migrated at least 2 cm.
4. Photograph the gel on transiluminator using short-wavelength UV irradiation (refer to safety precautions). Estimate the quantity of the DNA in the sample by comparing the intensity of the inflorescence with the standard DNA.
Spectrophotometry

This technique measures the total amount of nucleic acids in a sample (including NA, RNA, oligo-, and mononucleotides). Therefore, it is only useful for pure DNA preparations of a reasonable concentration.

Solutions
1X TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 or distilled water.

Method

1. Dilute a aliquot of the DNA sample in 1X TE or distilled water (usually in a ratio of 1:100; e.g., 5µl / 500µl) in microcuvate.
2. Determine the optical density at 260, 280 and 320 nm against a blank (1X TE or water).
3. Calculate the DNA concentration in the sample using the formula: 1.0 OD260 = 50 µg/ml (under standard conditions, e.g., 1 cm light path). The ratio of OD260 to OD280 provides some information about the purity of the DNA sample. Pure DNA preparations show as OD260 to OD280 ratio between 1.8 and 2.0. Contamination with, e.g., proteins results in lower values. The OD320 should be close to zero.
Agarose Gel Electrophoresis

In this technique, agarose is molten in electrophoresis buffer to yield a clear solution, cast into a gel mold, and allow solidifying. DNA, and DNA restriction fragments are applied to the gel, and a constant electric field is imposed. Under neutral or slightly alkalic conditions, DNA migrates toward the anode. Since the agarose matrix is acting a molecular sieve with pore sizes depending on the agarose concentration, restriction fragments up to about 20 kb are separated according to size. After finishing electrophoresis, DNA in the gels is stained with ethidium bromide, photographed, and further processed.

Since larger restriction fragments are frequently more informative for DNA fingerprinting purpose than small ones, relatively low gel concentrations are usually applied (between 0.7 and 1.2%). For example, a 0.7% agarose gel efficiently separates DNA fragments between 0.8 and 10 kb. Three buffer systems are generally used, i.e. TAE, TBE, and TPE. The resolving power of all three buffer systems is almost the identical. Though TAE is the most commonly used buffer it is probably the least advisable. The higher buffering capacity of TBE allows to use it in a 0.5X concentration.

Amounts of 5 to 10 µg of DNA per lane are usually sufficient for a fingerprinting experiment. Applying too much DNA may actually result in inferior banding patterns and thus should be avoided.

At least two or three lanes should be loaded with molecular weight markers, which are mixtures of restriction fragments of known size (0.2 to 2 µg, depending on gel size). After electrophoresis, these markers are visualized by staining, and their position documented by photography.

Solutions

Electrophoresis buffer (one of the three following):
1X TAE buffer: 40mM Tris-acetate, 1 mM EDTA, pH8.0 (adjust pH with glacial acetic acid)
0.5X TBE buffer : 45 mM tris=borate, 1 mM EDTA, pH 8.0 (adjust with boric acid)
1X TPE buffer: 90 mM Tris-phosphate, 2 mM EDTA, pH 8.0 (adjust pH with 85% phosphoric acid)

Note: Prepare electrophoresis buffers as 10 or 50X concentrated stock solutions and dilute prior to use.

Loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in electrophoresis buffer or water.
Molecular weight marker: DNA restriction fragments of known size (commercially available)
Agarose: e.g., 0.7% in electrophoresis buffer
Staining solution: 1 µg/ml ethidium bromide in electrophoresis buffer or water (refer to safety precautions)
Method

1. Suspend agarose (electrophoresis grade, e. g., Seakem LE) at the desired concentration in an appropriate amount of electrophoresis buffer in a bottle or flask (e. g., of agarose per 400ml of electrophoresis buffer yield a 0.7% gel). Flasks should be covered, and bottles should be loosely capped. Do not use aluminum foil!

2. Boil the suspension in a microwave oven for 2 to 4X2 min. Swirl the bottle in between. Continue until the agarose has dissolved. A good check for complete dissolution is the complete disappearance of lens-like particles.

3. Let the molten agarose cool to 60C, stirring helps to prevent uneven cooling. In the meantime, seal the open edges of the plastic tray supplied with the electrophoresis apparatus using tape. Insert a slot-forming comb. Band resolution is, to some extent, dependent on the shape of the teeth of the comb; sharp teeth yield sharp bands, but also allow less volume to be applied. Check that the teeth are not too close to the bottom of the gel mold. Fine holes in the bottom of a slot might allow samples to escape in an undesired direction.

4. Make sure that the gel mold is in a horizontal position. For some electrophoresis apparatus, the gel is best poured with the mold already in place. Carefully pour the agarose into the gel mold, remove small air bubbles with a pipette, and let the agarose solidify [usually 1h at room temperature or faster in a cold room (4C) or by turning on the water cooler system of the electrophoretic apparatus].

5. When the agarose is solid, carefully remove the comb and the trap, and insert the gel mold into an electrophoresis apparatus filled with buffer. Electrophoresis run best if there is not too much buffer on top (about 5mm). Remove air bubbles from the slots. Connect the apparatus to a power supply, and check whether it is working correctly (before applying the sample).

6. Add 0.2 vol of loading buffer to the DNA samples, mix, and centrifuge for a few seconds in a microfuge in order to collect the samples at the bottom of the tubes. The loading buffer adds color and provides a higher density to the samples, thus allowing their convenient application to the slots. Moreover, the dyes are moving toward the anode when voltage is applied (bromophenol blue runs about twice as fast as xylene cyanol), and this gives you an idea how far the electrophoresis has proceeded. Bromophenol blue usually run close to the front (at the same rate as linear DNA of 300bp in a 0.5X TBE).

7. Slowly load the samples into the submerged slots. Alternatively, samples may also be loaded into the tray slots (in this case, no loading buffer is needed). Buffer is then cautiously poured to the gel surface, and the gel is run for 20min before more buffer is added.

8. Turn on power supply, and start the electrophoresis, Running conditions are usually 1 to 2 V/cm (i. e., distance between the electrodes) for 24 to 48h. Longer runs give better resolution of larger fragments. To minimize diffusion, the gel should be preferably run in the cold (4C) or with water cooling.

9. After the run is completed, remove gel from the apparatus, and stain for 15 to 60 min (depending on the gel thickness) in a tray with staining solution. Then rinse the gel briefly in water, place it on a UV transilluminator, and photograph under UV light (302
nm). Use an orange or red filter, which only allows the fluorescence of the ethidium bromide-DNA complex to enter the camera. For photographic documentation, place a transparent or fluorescent ruler alongside the gel to align marker sizes in a gel with fragment sizes in forthcoming autoradiograms.
APPENDICES

Appendix I (CULTURE MEDIA)

Potato Dextrose Agar (PDA).

Preparation

Components:
- Slices Potato tissue     200g
- Dextrose                       20g (This could be replaced by sucrose 10g)
- Agar                            20g (This could be reduced according to the desired hardness degree of the solidified media)
- Distilled or Deionized water 1000ml (1000g)

Procedure:
Boil the potato slices inside 500ml of the distilled water until it gets to be soft, i.e. cooked. Strain the cooked potato though several layers of cheese cloth and collect 500ml of Potato Broth. At the same time, dissolve the Agar and Dextrose in another 500ml of distilled water. This is prepared by gradual addition of the agar powder to the heated water inside a large beaker (about 80-90°C) with continued stirring in order to avoid clumping of the agar and burning of the agar on the bottom surface of the beaker. Add the dextrose and continue stirring and maintaining the high temperature (above 70°C) until the entire agar get completely dissolved. At this stage the agar dextrose solution should be homogenous clear medium, i.e. it shows no tiny swelled agar particles. Mix both solutions, the Potato Broth (500ml) and the Agar Dextrose (500ml) to gather making 1000ml of PDA. Now this culture media is ready for sterilization.

Malt Extract Agar: MA (Malt Extract Agar); 30 g/l malt extract (Difco Laboratories), 15 g/l agar-agar, pH 7.0

Malt- Yeast Broth: 3 g/l malt extract, 3 g/l yeast extract, 5 g/l peptone, 10 g/l glucose, pH 7.5.

CMA (Cornmeal Agar): cornmeal extract, 15 g/l agar-agar, pH 6.0. Cornmeal extract is prepared as follows: boil 50g of ground maize grain wrapped in a cloth in a water bath. After simmering for 1 h, squeeze the extract through the cloth, and adjust to 1l with sterile water.

CZA (Czapek-Dox agar): Czapek-Dox broth (30g/l glucose, 5g/l yeast extract, 3g/l NaNO3, 1 g/l K2HPO4, 0.5 g/l MgSO4-7H2O, 0.01 g/l FeSO4-7H2O, 15 g/l agar-agar, pH 6.0 to 6.5.

Yeast-Peptone-Dextrose-Agar (YPDA) (yeast-peptone-dextrose agar): YPD medium (20 g/l glucose, 10 g/l yeast extract, 20 g/l peptone), 15 g/l agar-agar

Yeast Peptone Dextrose broth culture media (YPD broth):
20 g/l glucose, 10 g/l yeast extract, 20 g/l peptone.

Semi-Synthetic and Synthetic Culture Media
This culture media is basically made of salt solution (basic salts) as a source of the necessary nutrient elements for the fungal growth plus a source of carbon (any organic or inorganic carbon containing compounds). The media becomes a synthetic one (for example CZA) once it has all of its chemical constituents chemically defined. Such media is made of the basic
salts and glucose as the sole carbon source. Both kinds of culture media can be used as broth (liquid) or solidified with agar.

**Selective culture media**
This culture media is designed for the growth and/or suppression of certain group or individual microorganism. This selectivity can be accomplished to different degrees according to the constituents of the culture media itself, such as its reaction (pH), osmotic potential (salt concentration), inhibitors and promoters (antibiotics, specific growth promoters).

**Glucose- Yeast Extract- Peptone Media (GYPM):**
30 g/l glucose, 2 g/l yeast extract, 2 g/l peptone, mineral salts and trace elements mix stock [10 ml/l A, 10 ml/l B, 10 ml/l C1 ml/l D (see table below), pH 6.0. Solutions A and B are added before autoclaving the liquid of solid media. Solutions C and D are filter sterilized and added after autoclaving.

**Mineral Salts and Trace Elements Mix Stock**

<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Phosphate buffer</td>
<td>1.5 g/l KH2PO4, 0.2 g/l K2HPO4</td>
</tr>
<tr>
<td>B</td>
<td>Mineral Salt and trace elements solution</td>
<td>2.00 g/l NH4NO3, 0.4 g/l NaSO4, 0.15 g/l MgSO4-7H2O, 0.02 g/l MnSO4-4H2O, 0.02 g/l CuSO4-5H2O, 0.02 g/l ZnSO4-7H2O</td>
</tr>
<tr>
<td>C</td>
<td>Ferric solution</td>
<td>0.15 g/l Fe(NH4)2SO4</td>
</tr>
<tr>
<td>D</td>
<td>Calcium solution</td>
<td>1.10 g/l CaCl2</td>
</tr>
</tbody>
</table>

**CYM-Complete culture medium:**
20 g/l dextrose, 2 g/l yeast-extract, 2 g/l peptone, 1 g/l K2HPO4, 0.46 g/l KH2PO4, 0.5 g/l MgSO4-7H2O, pH 7.0.

**V8-juice Medium:**
200 ml/l V8-juice (unfiltered multivitamin juice), 3 g/l CaCO3, pH 7.2.

**Czapek-Dox medium:**
30 g/l glucose, 5 g/l yeast extract, 3 g/l NaNO3, 1 g/l K2HPO4, 0.5 g/l MgSO4-7H2O, 0.01 g/l FeSO4-7H2O, pH 6.0 to 6.5.

**Yeast Nitrogen Broth culture media (YNB):**
1.7 g/l yeast-nitrogen base without amino acids and ammonium sulfate, 5 g/l (NH4)2SO4, 20 g/l glucose.
Appendix II (SOLUTIONS)

- Washing solution: 0.1 M NaCl, 0.5% Triton X-100

- Extraction buffer: 25 mM Tris-HCl, 25 mM EDTA, 50 mM NaCl, 1% SDS, pH 8.0
  5M NaCl

- Extraction buffer: 1M Tris-HCl, 1.25 M NaCl, 0.25 M EDTA, pH 8.0

- Extraction buffer: 200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS
  3M sodium acetate, pH 5.2

- RNase A: 10 mg/ml (to inactivate contaminating DNases, the RNases has to be preincubated
  for 15 min at 100°C)

- Lysis buffer: 50 mM Tris-HCl, pH 7.2, 50 mM EDTA, 3% SDS, 1% β-mercaptoethanol

- Phenol (saturated with 1×TE, refer to safety precaution), Ratio phenol to chloroform to
  isoamyl alcohol (25: 24: 1)

- 1×TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

- Ethidium bromide: 10 μg/ml in water (refer to safety precautions)

- Electrophoresis buffer: TAB, TBE, or TPE (see previous sections)

- Gel-loading buffer: 30% glycerol, 1% SDS, 0.25% bromphenol blue

- Loading buffer: 0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol in
  electrophoresis buffer or water.

- Electrophoresis buffer (one of the three following):
  1× TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0 (adjust pH with glacial acetic acid)
  0.5× TBE buffer: 45 mM tris-borate, 1 mM EDTA, pH 8.0 (adjust with boric acid)
  1× TPE buffer: 90 mM Tris-phosphate, 2 mM EDTA, pH 8.0 (adjust pH with 85% phosphoric acid)

Note: Prepare electrophoresis buffers as 10 or 50× concentrated stock solutions and dilute
prior to use.