الجمهورية الجزائرية الديمقراطية الشعبية
وزارة التعليم العالي والبحث العلمي

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Ministry of Higher Education and Scientific Research

Academic year 2023/2024
Higher School of Biological Sciences of Oran
Second cycle Department

Educational handout of the workshop: Applied microbiology

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Pamphlet of applied microbiology
Level: 1st year of second cycle
Option: enzyme engineering
Specialty: Biotechnology
Stream: Biological sciences
Field: Natural and Life Sciences

2023 - 2024

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Presentation

This pamphlet is prepared and intended to the student of first year of the second cycle of biotechnology option **enzyme engineering** in Higher School of Biological Sciences of Oran.

Leader: Dr. SAIDI Yasmine

Instructors: Dr. BENYOUCEF Amel and Dr. MESSAOUI Hayet

Credits: 5

Rate: 3

Academic year: 2023 – 2024

Recommended prerequisites: biochemistry and microbiology

Applied microbiology workshop objectives

The main goal of this workshop is to provide students practical experiences and hands-out activities in applied microbiology. Therefore, the workshop is designed to demonstrate the practical value of experimental techniques in microbiology, specifically focusing on how they are used in producing enzymes using bacteria from the *Bacillus* genus. This subject will be taught through 7 sessions workshop during 12 weeks.

Teaching objectives

At the end of the workshop, students should be able to:

- Know and master the Good Practices in a microbiological Laboratory
- Develop microbiology concepts with easy-to-conduct experiments.
- Become familiar with basic laboratory instruments and understand the principles of measurements using these instruments through biochemical experiments.
- Master the basic techniques of isolating microorganisms.
- Master sampling techniques from soil, rhizosphere, and water.

Aimed techniques

- Isolation of bacteria belonging to the *Bacillus* genus on basic culture media.
- Detection of enzymatic activity on solid substrate-based medium using the lysis zone method.
- Macroscopic and microscopic identification of bacterial isolates.
- Biochemical identification.
- Bacteria growth measurement using a spectrophotometer at 600 nm.
- Measurement of the enzymatic activity of the enzyme α-amylase using the Miller DNSA method.

Applied microbiology workshop agenda

The workhop of applied microbiology is devided in 7 different sessions as described in the table below

Sessions	Intiteled	Program and objectives
Session 1 02/10/2023	Welcome and presentation of the workshop	- Teaching objectives - *Bacillus* overview - Hands-on acitivity - Tasks and assessement - Good Laboratory Practices - Laboratory Note Book
Session 2 08/10/2023	Sampling day	- The sampling will take part in the lake of Telamine (wilaya of Oran)
Session 3 **Group 1/3: 09/10/2023** **Group 2/4 : 16/10/2023**	Isolation of Bacteria	- Preparation of solutions and media - Isolation of bacteria from the different collected samples
Session 4 **Group 1/3: 23/10/2023** **Group 2/4: 30/10/2023**	Enzyme activity detection	- Subculturing the isolated bacteria on substrate-based media - Preparation of solutions and culture media
Session 5 **Group 1/3 : 06/11/2023** **Group 2/4: 13/11/2023**	Enzyme activity revelation Biochemical and physiological identification	- Enzymatic activities revelation. - Selection and Conservation of the best strains. - Morphological identification. - Subculturing of the selected strains on identification media
Session 6 **Group 1/3 : 20/11/2023** **Group 2/4 : 27/11/2023**	Enzyme kinetic and assays	- Culture of the identified strain on substrate-based liquid medium and monitoring of growth kinetics. - Measurement of the kinetics of enzyme activities by the DNS method.

Assessment

The assessment will be done as:

- A quiz at the end of each workshop concerning the work achieved that day. Each one counting for 10 %.
- The assiduity and participation will count for 5 % each.
- Final report written as an article counting for 50 %.

Good laboratory practices

A good laboratory practice in microbiology is essential to ensure accurate and reliable results, maintain a safe working environment. For that **some important rules must be respected and followed**:

- ✓ No lateness is tolerated, the student must come on time and prepared for the experiments
- ✓ A lab coat must be worn at all times in the microbiology laboratory.
- ✓ Headscarf must be tied and long hair must be tied back while working to avoid burning with open flames or inadvertent contamination.
- ✓ Open-toed shoes (sandals, flip-flops etc.) cannot be worn in the laboratory.
- ✓ It is strictly forbidden to eat, drink or smoke in the laboratory at any time.
- ✓ It is strictly forbidden to bring food or drink into the laboratory.
- ✓ It is strictly forbidden to store food or drink in laboratory, even for short time.
- ✓ Avoid all finger/hand-to-mouth contact.
- ✓ Wash hands, and wipe down bench area with disinfectant prior to working.
- ✓ Wash your hands at any time if you think you may have contaminated them.
- ✓ Wipe any surfaces or equipment with disinfectant immediately if you suspect contamination with living cultures.
- ✓ Avoid opening the windows during manipulations.
- ✓ Before leaving the laboratory for the day wipe down your bench area with disinfectant and then wash your hands and put everything back to its place.
- ✓ Treat all living cultures of microorganisms as potential pathogens.
- ✓ Avoid spilling or spreading the microorganisms.
- ✓ Place all used materials in the appropriate waste containers designated for cultures (to be autoclaved).
- ✓ Use the techniques specified by the instructor for handling microorganisms.
- ✓ If there is a spill notify your instructor immediately.
- ✓ To prevent contamination of personal items, books, coats, backpacks, etc., must be placed in the designated area and should not be kept at the laboratory bench.
- ✓ Never work alone in the laboratory
- ✓ You shall not use any chemicals in the laboratory without first reviewing their hazards.
- ✓ You shall not use any equipment in the laboratory without first reviewing proper use with your instructor.

- ✓ You shall notify your instructor or another faculty member of any spills, accidents or injuries that occur in the laboratory.

Introduction: *Bacillus* overview

What is *Bacillus*?

- Belonging to the family *Bacillaceae,* they are rod-shaped Gram-positive **Spore forming** bacteria, which are highly resistant, dormant structures that enable them to survive in harsh conditions, example: heat, cold, radiation, desiccation, and disinfectants
- **Ubiquitous** commonly found in various environments, including soil, water, and the gastrointestinal tracts of animals.
- Some of them are **pathogenic** (*B. anthracis* causing anthrax and *B. cereus* causing poisoning food).

Why working with *Bacillus*?

- They are known for their diverse metabolic abilities (**thanks to their enzymes**) and are of significant interest in scientific research, industrial applications, and medicine.
- The members of the Association for General and Applied Microbiology (VAAM) decided that the bacteria of the year 2023 is *Bacillus subtilis.*
- It has several applications:
  - ✓ **Biotechnological Applications**
    - ➢ Industrial uses in enzyme production.
    - ➢ Role in bioremediation.
  - ✓ **Medical and Pharmaceutical Applications**
    - ➢ *Bacillus* as probiotics and therapeutic agents.
    - ➢ Antibiotics and antimicrobial peptides.
  - ✓ **Food and Beverage Industry**
    - ➢ *Bacillus* in food preservation and fermentation.
    - ➢ Safety and regulatory aspects.

References

- Myrsini N. Kakagianni (2022) Spoilage Organisms: *Geobacillus stearothermophilus.* In Paul L.H. MSweeney and John P. McNamara (Ed.) *Encycopedia of dairy Sciences third edition* (page 384-393). Academic Press.

Hands-on activities

The different sessions of the applied microbiology workshop will take place in the field (for the sampling day) and in the microbiology laboratory situated in the Higher School of Biological Sciences of Oran (for the 5^{th} other sessions)

WORKSHOP 1: sampling day

1. **Purpose of the workshop**
   - To learn how to do sampling from soil, water, and rhizosphere.
   - To learn how to use correctly the tools to ensure aseptic collection.
   - To learn how to use the in-situ measurement instrument (thermometer, pH-meter)
   - To learn how to choose the right place for sampling to obtain a representative sample that reflects the true diversity of microorganisms in the soil.

2. **Sampling site**
   - The sampling will take place in Lake of Telamine (Gdyel oran) on the October 08th, 2023 for the 4 groups at the same time
   - Three different sampling will be done on this site:
     - ➢ Collection of soil
     - ➢ Collection of rhizosphere
     - ➢ Collection of Water

➢ **Lake of Telamine**

The lake of Telamine in East of Oran (Algeria) 7 km far from the industrial zone of Hassi Ameur covers an area of approximatively 2399 ha (Fig. 1). The lake of Telamine (35°43'35.9"N 0°23'28.7"W) is the second largest basin in Oran after that of the sebkha Arzew, with a length of 6 km and 1 km of mean width and has a mean elevation of (84.5 m) above sea leavel (Belguermi *et al.,* 2014).

Figure 1: Representative map of lake of Telamine (Google maps 2023)

3. Sampling material

- Latex gloves
- Spade (4)
- Sterile spatula (8)
- Bunsen burner (1)
- Petri dishes (40)
- Sterile bottles (40)
- Cooler bag with ice packs
- Paper towel
- pH meter
- Thermometer

4. Sampling method

Wetland soil is known for its range of species, which proliferate in the varied hydrological conditions and vegetation. When studying the community leaving there it is important to collect samples, from bulk soil, rhizosphere and water, ensuring that the collection process is both random and representative. By following this approach, we can ensure that the data obtained aligns with the objectives of our study (Aixin Hou and Henry N. Williams, 2013).

4.1. Collection of soil

- Process
  - Record the GPS position of the sampling location.
  - Codify the Petri dish.
  - Dig a small area of the chosen part of the soil using a small spade.
  - Avoid touching the soil with your hands.
  - Take the sample using a spatula previously sterilized using the portable Bunsen burner and put approximately 10 g in the Petri dish.
  - Place directly in the cooler bag with ice packs to maintain a low temperature during transport to the laboratory.

4.2. Collection of rhizosphere

- Choose a plant, take a photo of it and record the GPS position of the sampling location.
- Codify the Petri dish.

- Dig a little next to the plant until you reach its roots using a spade.
- Avoid touching the soil with your hands.
- Take the sample using a spatula previously sterilized using the portable Bunsen burner and put approximately 10 g in the Petri dish.
- Place directly in the cooler bag with ice packs to maintain a low temperature during transport to the laboratory.

4.3. Collection of water

- Record the GPS position of the sampling location.
- Code the collection bottle.
- Immerse the bottle to a depth of approximately 10 cm, fill it halfway and close it immediately.
- Place directly in the cooler bag with ice packs to maintain a low temperature during transport to the laboratory.

5. References

- Belguermi, A.; Belhaouari, B.; Boudaoud, K.; Boutiba, Z. Physico-Chemical Characteristics of water and Ornithological Assessment of LakeTelamine (Algeria). International Journal of Sciences: Basic and Applied Research 15 (2014) 1-8.
- Hou, A., Williams, H.N. (2013). Methods for Sampling and Analyzing Wetland Soil Bacterial Community. In: Anderson, J., Davis, C. (eds) Wetland Techniques. Springer, Dordrecht. https://doi.org/10.1007/978-94-007-6931-1_2

WORKSHOP 2: Media preparation and isolation of bacteria

1. Purpose of the workshop

- To learn how to describe the different media culture used and their component
- To learn how to prepare and transfer each media culture used
- To learn how to prepare different form of media culture (broth, agar slant, agar deep tube, agar plate)
- To learn how to sterilize media
- To learn how to isolate *Bacillus* species using the dilution method

2. Place of the workshop

In microbiology laboratory

3. Material needed

- Screw tubes
- Petri dishes
- Spatula
- Bottles
- stirrer/heating plate with stirring bar
- screw tubes
- Test-tube rack or wire basket
- Micropipette
- Water bath
- Paper towel
- Alcohol 70 °C
- Vortex
- Autoclave
- Heat-proof gloves
- Erlenmeyer flasks (250 mL and 500 mL)
- Beakers
- Balance
- Bleach
- Distilled water

4. Method used

4.1. Media preparation

Similar to all living organisms, microorganisms depend on nutrients for their growth and survival. Microbiological media, consist of a blend of water and essential nutrients essential for microbial growth. It can be either prepared from dehydrated media or by mixing different weighed basic ingredients that are dissolved in distilled water

Microbiological media can be prepared in various forms:

- **Liquid broth:** It doesn't contain agar. It is used for inoculum preparation, different biochemical tests

- **Agar slants:** where agar is tilted during solidification to increase the surface area
- **Agar deep tube**: typically inoculated by stabbing agar with an inoculation needle
- **Petri plates:** offering a larger surface area for microbial growth on the agar's surface. Allow to appreciate colony morphology and pigmentation

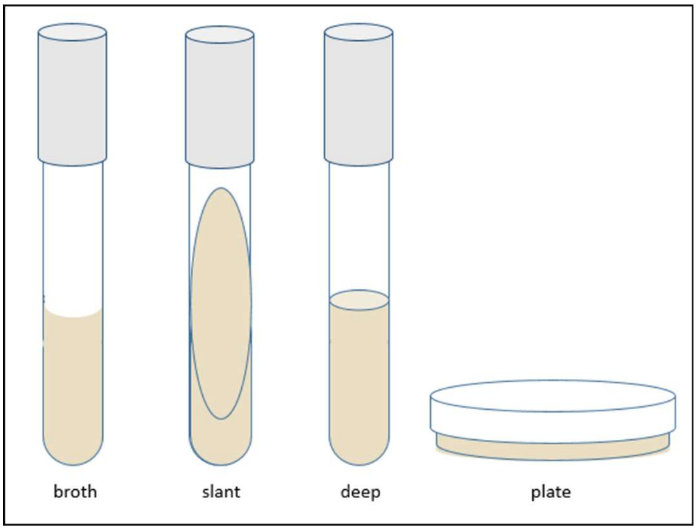

Figure 2: Different way to prepare culture media

4.1.1. Preparation of Nutrient broth and agar

The preparation of nutrient agar will be done by using dehydrated media by following the instruction of preparation described on the Label to prepare 1L. To obtain the solid media, 2 % of agar will be added and heated until the medium starts simmering or boiling.

- The pH is adjusted to the pH of the sample.
- The media is sterilized by autoclaving at 121° C for 20 minutes.

The nutrient agar will be used

- To fill Petri dishes for the *Bacillus* isolation.
- For the substrate-based media to look for some *Bacillus* enzymes.
- To prepare agar slant in tube in order to conserve the isolates.

4.1.2. Preparation of substrate-based media

✓ **1 % Starch agar media (to look for amylase)**

- 360 ml nutrient agar
- 40 ml of starch at 10 % (10 g of soluble starch in 100 ml of distilled water)
  - The pH is adjusted to the pH of the sample
  - The media is sterilized by autoclaving at 121° C for 20 minutes

- ✓ **1 % skimmed milk Agar media (to look for caseinase)**
  - ➢ 200 ml agar double concentrated (6 g of agar in 200 ml of water)
    - The media is sterilized by autoclaving at 121° C for 20 minutes
  - ➢ At the moment of use 200 ml Skimmed milk UHT is added to the supercooled media aseptically
- ✓ **Tween media (to look for lipase)**
  - ➢ 04 g of peptone
  - ➢ 02 g of sodium chloride (NaCl)
  - ➢ 0.4 g of calcium chloride ($CaCl_2$, $2H_2O$)
  - ➢ 10 g agar
  - ➢ 400 ml of distilled water
    - The media is sterilized by autoclaving at 121° C for 20 minutes

At the moment of use 6,5 ml of autoclaved tween® 80 are added to the supercooled media aseptically.

4.2. Preparation of solution

4.2.1. Saline solution

Used to prepare the decimal solution of each sample.

- ➢ 9 g of NaCl
- ➢ 1 L of distilled water
  - The solution is sterilized by autoclaving at 120 °C during 20 minutes.

4.2.2. Starch at 10 %

10 g of soluble starch in 100 ml of distilled water.

4.3. Isolation of bacteria

Dilution is a process that involves reducing the concentration of a substance in a solution. Decimal dilutions of the samples to be analyzed are carried out using a 1000 µl micropipette

Process:

- A stock solution is created by mixing 1 g of soil or rhizosphere (1 ml of tested water) with 9 ml of saline water. Mix with the vortex.

- From this suspension, 1 ml is taken and transferred to a tube containing 9 ml of sterile saline solution, resulting dilution 10^{-1}. Mix gently.
- 1 mL of this dilution dis taken and added to the next tube (10^{-2}), mix gently.
- This operation is repeated until reaching the dilution 10^{-6}.
- The two last dilutions 10^{-5} and 10^{-6} are subjected to heat treatment in a water bath at 80 °C for 10 minutes before being inoculated onto the nutrient agar medium. Then cooled in cold water.
- This pretreatment serves to kill the vegetative forms and selectively isolate the spore forms of *Bacillus.*
- Then, the surface of each plate is inoculated with a drop from each dilution (10^{-5} and 10^{-6}) using a sterile platinum loop (using a zig zag streak).
- The inoculation has to be done carefully to not compromise the agar media.
- The incubation is done at 37 °C for 24 h.

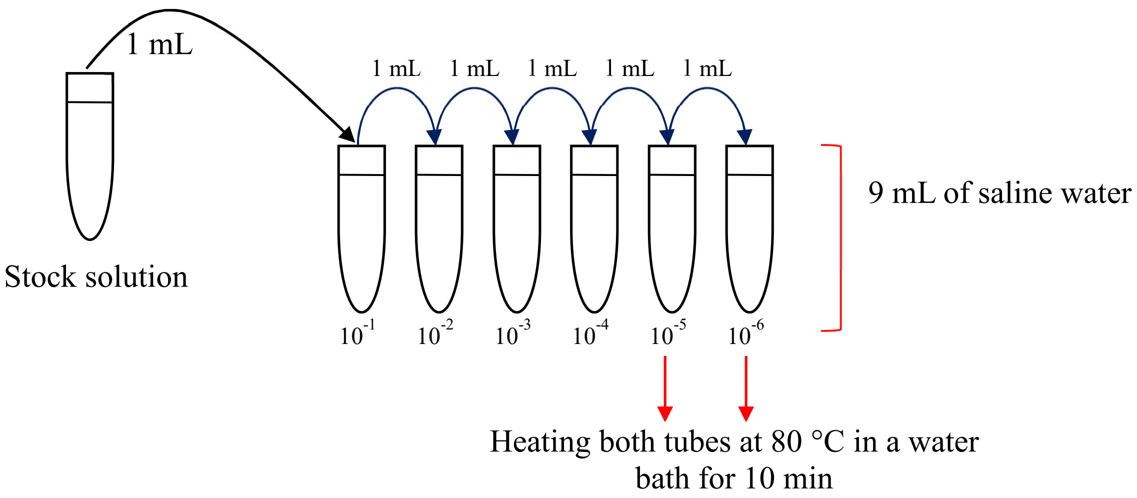

Figure 2: Preparation of decimal dilution from soil, rhizosphere or water sample

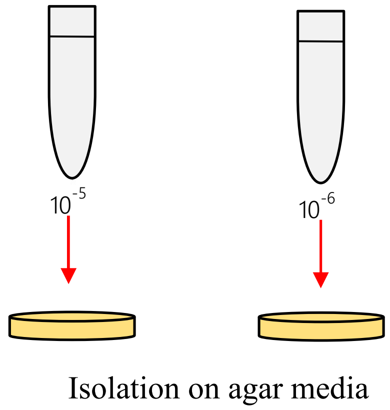

Figure 3: Isolation of bacteria on agar media plate using Nutrient agar

Workshop 3: Media preparation, isolates pre-identification and detection of enzymatic activity

1. Purpose of the workshop

- Learning how to describe the different media culture used and their component.
- Learning how to prepare and transfer each media culture used.
- Learning the importance of purified culture.
- Learning how to describe colony morphology.
- Learning how to pre-identify isolates
- Properly prepare a bacterial smear for accurate staining.
- Learn Gram staining
- Detecting of the enzymatic activity using substrate-based media.

2. Place of the workshop

In microbiology laboratory

3. Material and reagents needed

- Screw tubes
- Petri dishes
- Spatula
- Bottles
- stirrer/heating plate with stirring bar
- screw tubes
- Test-tube rack or wire basket
- Micropipette
- Vortex
- Water bath
- Paper towel
- Alcohol 70 °C
- Crystal violet
- Distilled water
- Autoclave
- Heat-proof gloves
- Erlenmeyer flasks (250 mL and 500 mL)
- Beakers
- Balance
- Glass slide
- Wood clamp
- Microscope
- Iodine mordant
- Carbolfuchsine
- Hydrogen peroxide
- Bleach

4. Method used

4.1. Media preparation

4.1.1. Nutrient broth

Read and follow the instruction of preparation described on the Label to prepare 1L. It will be devided in 3 portions

- The 1st portion, the medium will be alkalinized to pH 10.
- The 2nd portion, 15 % of NaCl will be added to the medium (and the pH will be adjusted to the pH of the sample.
- The 3rd portion, its pH will be adjusted to the pH of the sample without adding anything.
- The media are sterilized by autoclaving at 121 °C for 20 minutes.

4.1.2. Nutrient agar

Read and follow the instruction of preparation described on the Label. To obtain the solid media, 2 % of agar will be added and heated until the medium starts simmering or boiling.

- The pH is adjusted to the pH of the sample.
- The medium is sterilized by autoclaving at 121 °C for 20 minutes.

4.1.3. Meat liver agar

Read and follow the instruction of preparation described on the Label to prepare 1L.

- The pH is adjusted to a pH 7.6.
- The medium is sterilized by autoclaving at 121 °C during 20 minutes.

4.1.4. Citrate Simmons agar

Read and follow the instruction of preparation described on the Label to prepare 1L.

- The pH is adjusted to a pH 6.8.
- The medium is sterilized by autoclaving at 121 °C during 20 minutes.

4.1.5. Lecithinase production medium

- 1 % Tryptone
- 0.1 % Disodium phosphate (Na_2HPO_4)
- 0.1 % Potassium dihydrogen phosphate (KH_2PO_4)
- 0.01 % Magnesium sulfate heptahydrate ($MgSO_4$, $7H_2O$)
- 0.2 % Sodium chloride (NaCl)

- 0.2 % Glucose
- Distilled water
- pH adjusted to 7.6
- After autoclaving 1.5 % egg yolk is added to the broth in a sterile area then the medium is mixed and refrigerated for 24 hours at 4 °C. The top phase was used as medium.

4.2. Purification of the isolates

Purification is a crucial and delicate step in the bacterial characterization process. After the initial inoculation of the sample on Petri dishes, various colonies are obtained, and the accuracy of the identification results depends on this step. The strains were purified through successive subcultures on nutrient agar (NA), using the method of zig zag streak **(fig. 5)** and in order to reduce the cell density on the surface of the plate, the T-Streak or a quadrant streak technique was used **(fig. 6),** until colonies with the same appearance and color were obtained in the Petri dishes (Frunklund, 2018).

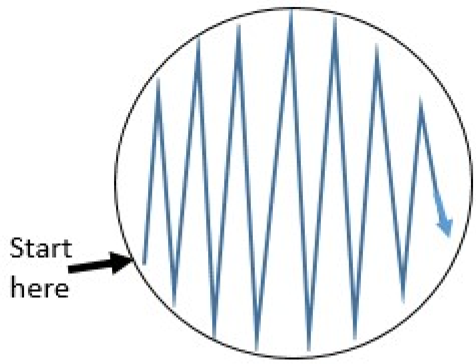

Figure 5: Zig zag inoculation pattern

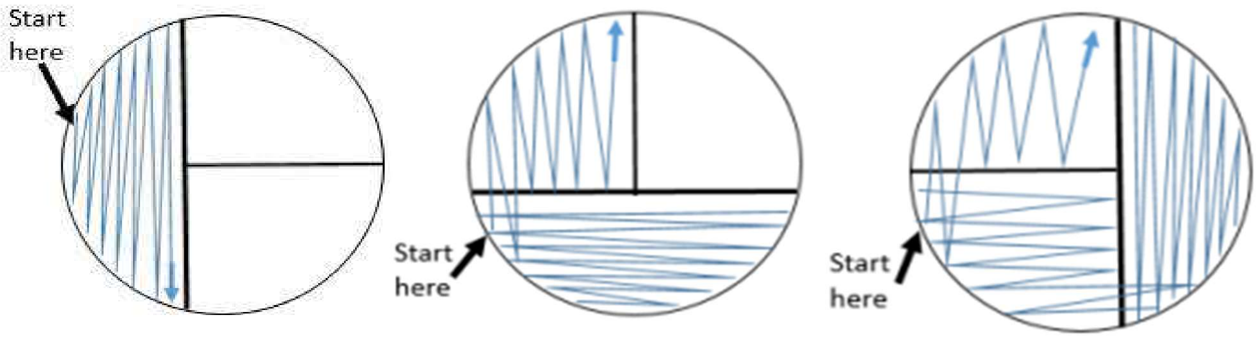

Figure 6: steps for quadrant streak pattern

4.3. Pre-identification of the isolates

This characterization is carried out for the primary identification of isolates based on the macroscopic and microscopic observation after Gram coloration and catalase test

4.3.1. Macroscopic observation of the colonies

There are seven basic characteristics to identify bacterial colony morphology (Petersen 2016). We can describe the morphology of a bacterial colony by looking into the aspects like:

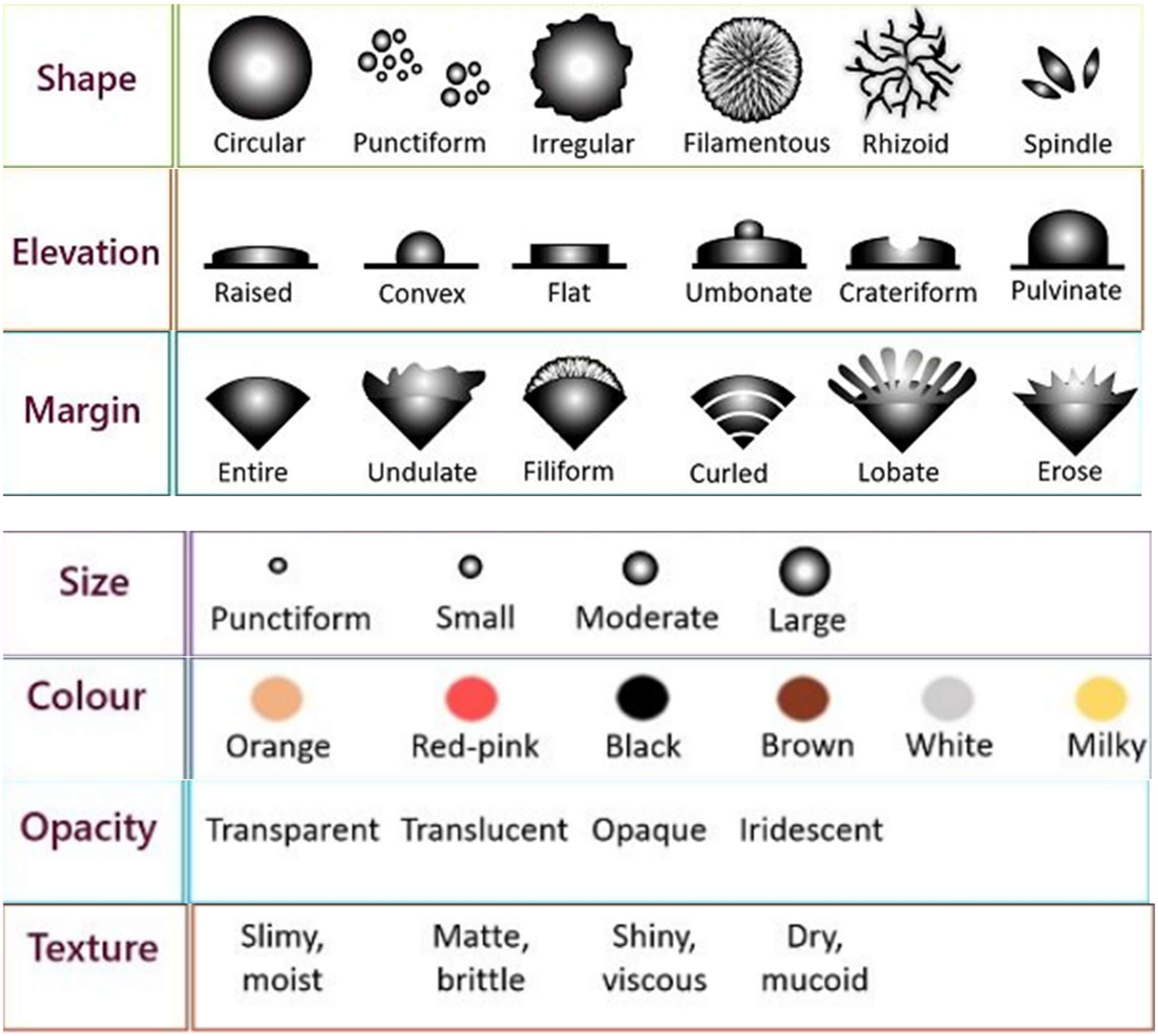

Figure 7: Different description of bacterial colonies

4.3.2. Microscopic observation

The microscopic observation is done after the Gram stain of a heat-fixed smears of isolates.

4.3.2.1. Smear preparation

- In a bacterial smear that has been properly processed, the bacteria are evenly spread out on the slide in such a concentration that they are adequately separated from one another.
- In making a smear, bacteria from either a **broth culture** or an **agar slant** or **plate** may be used. If a slant or plate is used, a small amount of bacterial growth is transferred to a drop of water on a glass slide.

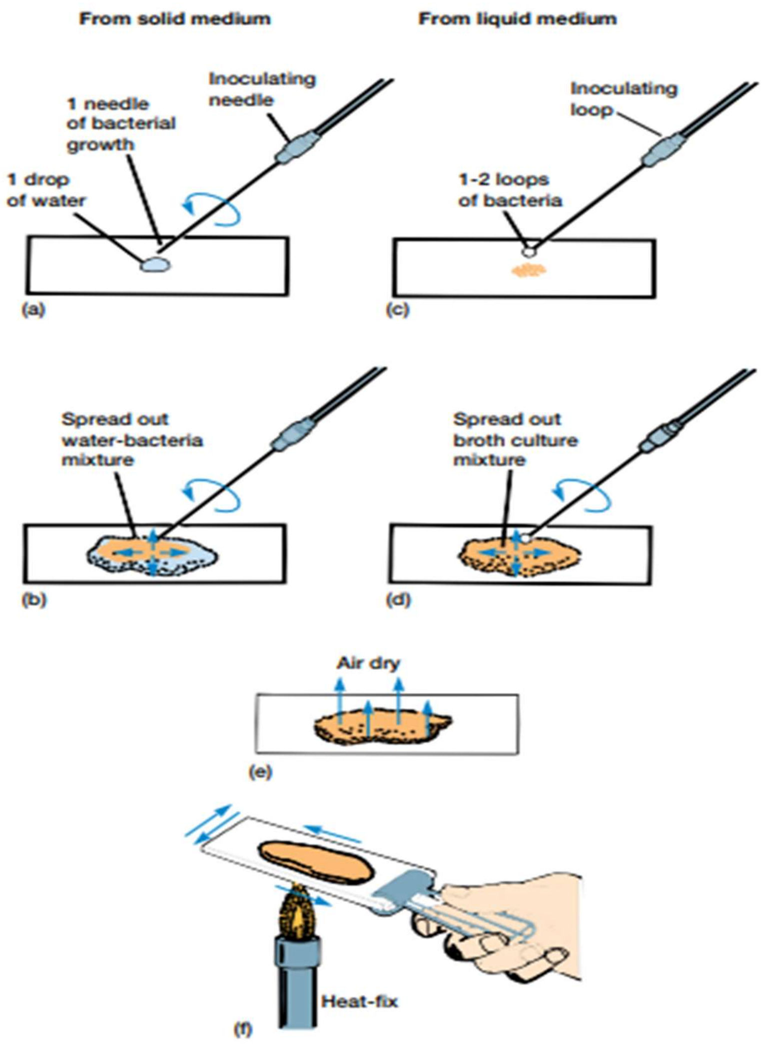

Figure 8: Heat-fixed smear preparation

4.3.2.2. Gram staining

The Gram staining is based on the difference in the permeability of bacteria to alcohol, and thus their ability to retain a primary stain (crystal violet) in their cytoplasm. This difference in permeability allows bacteria to be classified into two major groups, Gram-negative or Gram-positive, to which strains of the *Bacillus* genus generally belong.

- First, crystal violet, **the primary stain**, is applied to a heat-fixed smear, giving all of the cells a purple color
- Next, Gram's iodine, a **mordant**, is added. A mordant is a substance used to set or stabilize stains
- Next, a **decolorizing agent** is added, usually ethanol or an acetone/ethanol solution. Cells that have thick peptidoglycan layers in their cell walls are much less affected by the decolorizing agent; they generally retain the crystal violet dye and remain purple. These are termed **Gram positive**.
- Finally, a secondary **counterstain,** usually carbolfuchsin, is added. This, stains the decolorized cells pink.

4.3.3. Catalase test

- Add 1 drop of 3 % hydrogen peroxide on a bacterial colony.
- The appearance of effervescence indicates the degradation of hydrogen peroxide under the action of the enzyme catalase and the release of oxygen.

4.4. Detection of enzymatic activity

In this workshop, 3 enzymes will be researched: α-amylase, caseinase, and lipase using different media culture:

- α-amylase: using 1 % starch agar media
- Caseinase: using skimmed milk Agar media
- Lipase: using Tween® media

Transfer morphologically different colonies to the different substrate-media plates using the platinum loop with only one central streak or with the method of multipoint deposit point. Incubate the different plates at 37 °C during 24 – 48 h.

Figure 9: (a): Central streak on agar plate. (b): multipoint deposit colony.

5. References

- D. Breakwell, C. Woolverton, B. MacDonald, K. Smith and R. Robison, 2007. Colony morphology Protocol. American society of microbiology.
- Franklund, C. (n.d.). Microbiology Laboratory Manual, Observing and Recording the Microbial World. Farris State University. Retrieved 2018, from https://github.com/WeeBeasties/microbiologylaboratory-manual.
- John P. Harley Lansing M. Prescott (2002). Laboratory Exercises in Microbiology, 5th ed. The McGrawí Hill Companies.
- Petersen, J. a. (2016). Laboratory Excercises in Microbiology: Discovering the Unseen World Through Hands-On Investigation. CUNY Academic Works. Retrieved from http://academicworks.cuny.edu/qb_oers/16

Workshop 4: Enzymatic activity revelation and identification of best isolates

1. Purpose of the workshop

- To learn how the enzymatic activity is revealed
- To learn the interpretation of different media for biochemical identification and physiological characterization
- To streak an isolate in purpose to purify it.

2. Place of the workshop

In microbiology laboratory

3. Material needed

- Screw tubes
- Petri dishes
- Spatula
- Bottles
- stirrer/heating plate with stirring bar
- screw tubes
- Test-tube rack or wire basket
- Micropipette
- Water bath
- Paper towel
- Distilled water
- Vortex
- Autoclave
- Heat-proof gloves
- Erlenmeyer flasks (250 mL and 500 mL)
- Beakers
- Balance
- Alcohol 70 °C
- Bleach

4. Method used

4.1. Revelation of enzymatic activity

After 48 h of incubation, the revelation of the enzymatic is done:

4.1.1. α-Amylase

The hydrolysis of starch, results in appearance of a clear zone around the colony, demonstrating the degradation of casein. If it appears the diameter is measured (Itsouhou et *al.,* 2020).

4.1.2. Caseinase

For caseinase activity, also a clear zone appears demonstrating the degradation of casein and the diameter is measured (De VOS *et al.,* 2009).

4.1.3. Lipase

Lipase leads to the production of fatty acids that link with the calcium chloride (present if the medium), resulting in the formation of a white opaque border surrounding the colony (Ramnath *et al.,* 2017).

4.2. Biochemical identification

The Gram positive, catalase positive bacteria that show the best enzymatic profile are identified using the classical method of identification (phenotypical identification) using different media:

4.2.1. Simmons citrate medium

It allows the determination of the bacteria's capacity to use citrate as the only source of carbon for its energy needs. It involves streaking on the slant of Simmons citrate agar using Pasteur pipette. The method consists on the streaking on the surface of the slant of Simmons citrate agar using a closed Pasteur pipette. Then the tubes are incubated for 24 – 48 h at 37 °C. Examine the slant cultures for the presence or absence of growth and for any change in color from green to blue (due to an alkalinization of the medium) (Harley et Prescott, 2002).

4.2.2. Respiratory test using meat liver agar medium

After a regeneration of the medium during 30 min in a water bath, the isolates are streaked using a closed Pasteur pipette by spiraling upward into the agar. The tubes are incubated during 24 – 48 h at 37 °C.

The respiratory type is determined by defining the position of the culture in the tube:

- Surface culture: Strict aerobic type.
- Culture everywhere except on the surface: Strict anaerobic type.
- Culture throughout the tube: Facultative aerobe-anaerobe type.
- Culture near the surface of the tube: Microaerophilic type.

4.2.3. Lecithinase test

- Isolates are cultured on egg yolk-based medium and incubated at 37 °C for 48h – 72h.

- The presence of a white precipitate at the bottom of the tubes indicates the production of lecithinase.

4.3. Physiological identification

- Growth on alkaline medium: The purified isolates are cultured on nutrient broth at pH 10, and incubated for 48 to 72 hours at 37 °C.
- Growth on saline medium: The isolates are cultured on 15 % NaCl medium and incubated for 48 to 72 hours at 37 °C.
- Growth at 60 °C: isolates are grown in nutrient broth for 48 - 72h at 60 °C.

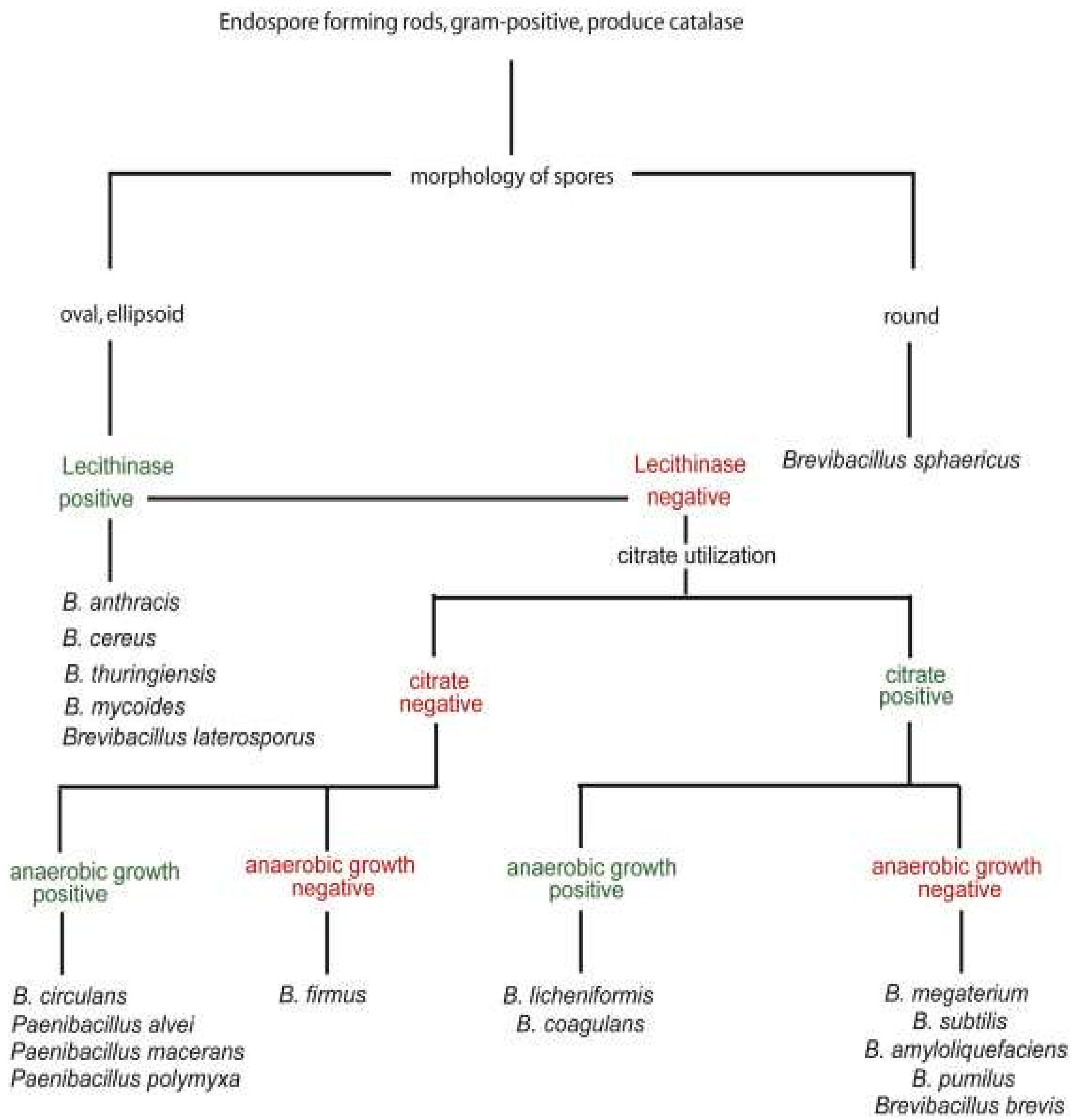

Figure 10: Scheme for an early differentiation of bacilli by their morphologic and phenotypic features

5. References

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Workshop 5: different preparations for enzyme assays and bacterial growth kinetic

1. Purpose of the workshop

- To learn the composition of media to produce enzyme.
- To identify the isolates obtained with biochemical and physiological method

2. Place of the workshop

In microbiology laboratory

3. Material and reagents needed

- Test-tubes
- Spectrophotometer and cuvettes
- Incubator
- water bath
- Bunsen burner
- Eppendorf microtubes 1.5 ml
- Plastic tubes (15ml)
- Beakers
- test tubes
- Micropipettes 1000 µl
- Sterile tips rack
- Sterile distilled water
- Potassium buffer 50 mM pH7
- Enzyme production medium
- Wheat bran
- Glucose
- Paper towel
- Alcohol 70 °C
- Bleach

4. Method used

4.1. Media preparation

4.1.1. Enzyme production media

- 0.173 % Dipotassium hydrogen phosphate (K_2HPO_4)
- 0.068 % Potassium dihydrogen phosphate (KH_2PO_4)
- 0.01 % Magnesium sulfate heptahydrates ($MgSO_4$, $7H_2O$)
- 0.4 % Sodium chloride (NaCl)
- 0.003 % Ferrous sulfate heptahydrate ($FeSO_4$, $7H_2O$)
- 0.1 % Ammonium nitrate (NH_4NO_3)
- 0.002 % Calcium chloride ($CaCl_2$, $2H_2O$)
- 0.5 % of wheat bran
- Distilled water

The pH is adjusted to a pH 7.6.

The medium is sterilized by autoclaving at 121 °C during 20 minutes.

4.2. Reagents preparation

4.2.1. DNSA reagent (3,5-dinitrocalicycilic acid)

- 10 g DNSA
- 16 g NaOH
- 5 g melted Phenol
- 5 g Sodium sulfite
- 300 g potassium sodium tartrate
- 1 L distilled water

4.2.2. Potassium phosphate buffer, 50 mM, pH 7.0

- 1.26 g of Monopotassium phosphate (KH_2PO_4)
- 1.86 g of Dipotassium phosphate (K_2HPO_4)
- 1 L distilled water.

4.2.3. Buffered starch 1%

- 1 g of starch
- 100 mL of Potassium phosphate buffer, 50 mM, pH 7.0.

N.B: each prepared reagent is sterilized by autoclaving at 121 °C during 20 min.

Workshop 6: Enzyme assays and bacterial growth kinetic

1. Purpose of the workshop

- To learn how to produce enzyme.
- To learn how to extract enzyme.
- To know the spectrophotometer principles.
- To Create a calibration curve using predetermined quantities of glucose.
- To learn how to use the calibration curve in order to figure out the amylase enzyme activity.

2. Place of the workshop

In microbiology laboratory

3. Material used

- Test-tubes
- Spectrophotometer and cuvettes
- Incubator
- Water bath
- Bunsen burner
- Eppendorf microtubes 1.5 ml
- Plastic tubes (15ml)
- Test tubes
- Micropipettes 1000 µl
- Sterile tips rack
- Sterile distilled water
- Enzyme production medium
- Nutrient Broth (NB)
- DNSA reagent
- Buffered solution of starch 1 %
- Glucose
- Paper towel
- Alcohol 70 °C
- Bleach

4. Method used

4.1. Preparation of overnight culture in enzyme production media

- 10 ml of the enzyme production media is inoculated with 0.1 ml of each selected strain.
- The incubation is done under shaking for 24 h at 37 °C.
- 10 ml of the enzyme production is incubated for 24 h at 37 °C without being inoculated (to be used as blank).

4.2. Enzyme extraction

After 24 h of incubation, the cultures are centrifuged at 5000 rpm for 15 min, where the supernatant is transferred to sterile tubes.

4.3. Preparation for the calibration curve

Prepare a calibration curve following the instructions:

- Prepare a stock solution of glucose with a concentration of 2 mg/ml.
- Prepare standards containing glucose in increasing concentrations from 0.4 to 1.2 ml/mg in a final volume of 1 ml of distilled water **(table 1).**
- Mix each 0.5 ml of each concentration with 0.5 ml of DNSA reagent.
- The blank is prepared by mixing 0.5 ml of distilled water with 0.5 ml of DNSA reagent.
- Incubate at 100 °C for 5-10 min.
- Read the absorbance at 540 nm.
- Plot the standard curve of the absorbance (at 540 nm) on "Y" axis versus the concentration of glucose on the "X" axis

Table 1: Preparation of calibration curve of glucose

Glucose (mg/ ml)	**0**	**0.4**	**0.8**	**1.2**	**1.6**
Glucose stock solution (ml)	0	0.2	0.4	0.6	0.8
Distilled water (ml)	1	0.8	0.6	0.4	0.2

4.4. Amylase assay

4.4.1. Assay principle

The DNSA method is used to estimate the concentration of reducing sugars in a sample (all the monosaccharides and some disaccharides are considered as reducing sugars).

The reducing sugars contains free carbonyl group, having the property to reduce many reagents. When the alkaline solution of **3,5-dinitrocalicycilic acid (DNSA)** reacts with reducing sugar (ex: glucose), it is converted into **3-amino-5-nitrosalicylic acid (ANSA)** with orange color having an absorbance maximum of 540 nm (Miller, 1959).

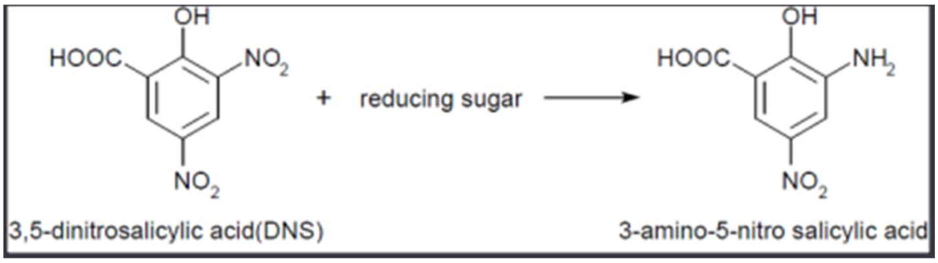

Figure 11: Chemical reaction for DNSA method

4.4.2. Procedure

- The buffered starch at 1 % is prewarmed at 37 °C.
- 3 ml of each supernatant is mixed with 3 ml of the buffered starch 1 % and incubated during 30 min at 37 °C.
- Samples of 0.5 ml are taken each 5 min (from T_0 to T_{30}).
- 0.5 ml of DNSA reagent is added to each tube (samples and blank).
- The mix is Incubated for 5-10 min at 100 °C (to stop the enzymatic reaction).
- The absorbances are read at 540 nm.
- Record the value "X" of unknown from graph corresponding to the OD reading of the test samples.

4.5. Growth kinetic of the isolated strains

The principle is that the optical density (OD) of a bacterial culture, measured through absorbance, increases as the number of bacteria in the culture grows.

The absorbance is in correlation with the growth: an increase in absorbance is typically interpreted as an elevation in bacterial density and thus in bacterial growth.

4.5.1. Procedure

4.5.1.1. Preparation of overnight culture in enzyme production media

- 10 ml of the enzyme production media is inoculated with 0.1 ml of each selected strain.
- The incubation is done under shaking for 24 h at 37 °C.

4.5.1.2. Kinetic growth

- 10 ml of the Nutrient broth is inoculated with 0.1 ml of the overnight culture.
- The incubation is done during 6h at 37 °C.
- Each one hour, a sample of the culture is taken, and the absorbance is measured using a spectrophotometer at a specific wavelength of 600 nm.
- Plot a curve of the absorbances at 600 nm in the "Y" axis versus the time of sampling.

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