الجمهورية الجزائرية الديمقراطية الشعبية

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وزارة التعليم العالي والبحث العلمي

MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH

Academic Year : 2024/2025

Higher School of Biological Sciences of Oran (HSBSO)

Department of Second Cycle

Teaching Handout

Course Title

WORKSHOP OF ENZYME ENGINEERING

Level : 2nd Year of Second ycle
Speciality : Molecular Biology
Stream: Biological Sciences
Field: Natural and Life Sciences

Instructor

Dr. Imène KERSANI

Course Taught During the Academic Years
2023/2024
2024/2025

Foreword

Enzyme engineering, also known as protein engineering, refers to the process of altering the amino acid sequence of proteins or enzymes through recombinant DNA mutation to enhance their properties. This modification aims to adjust the catalytic activity of individual enzymes, facilitating the production of new metabolites and ensuring the formation of novel reaction pathways.

This handout concerns the study of Methodology Teaching Unit 2 (MTU 2), designed for students in the second year of the second cycle at HSBSO (Speiality : Molecular Biology), during the third semester. It aligns with the official annual curriculum established by HSBSO, as stipulated in the ministerial decree of the Minister of Higher Education and Scientific Research.

This educational resource is intended for students with prior knowledge in biochemistry, microbiology, and fundamental enzymology. It aims to deepen this foundation by introducing advanced methodologies and concepts in enzyme engineering, preparing students for both complex theoretical frameworks and practical laboratory applications.

The workshop focuses on developing key competencies such as the optimization of enzyme production conditions, enzyme capture, purification through chromatography, and the establishment and analysis of a purification table. These targeted skills are essential for understanding enzyme behavior, enhancing process efficiency, and supporting downstream biotechnological applications.

The enzyme engineering workshop is divided into six sessions :

- ♣ **Session 1 : Preparation of solutions and culture media,** which includes an opening lecture outlining the rules and guidelines from instructors regarding laboratory safety. This session emphasizes critical safety reminders for students to prevent accidents while preparing the necessary solutions and culture media for the upcoming sessions.
- ♣ **Session 2: Production of α-amylase enzyme by fermentation,** is centered on identifying the ability of *Bacillus subtilis* strain to secrete α-amylase into the extracellular environment and to hydrolyze starch, as evidenced by the addition of Lugol's iodine solution. The workshop will also cover the production of this bacterial enzyme via submerged fermentation in a suitable production medium. Amylolytic

activity and protein content are determined using the methods of **Miller (1959)** and **Bradford (1976)**, respectively.

♣ **Session 3: Purification of α-amylase enzyme,** involves purifying the bacterial enzyme while eliminating impurities through fractional precipitation with ammonium sulfate, followed by dialysis and chromatography techniques, including size exclusion and affinity chromatography.

♣ **Session 4 : Evaluation of purification,** focuses on assessing the purity of the α-amylase enzyme through electrophoresis, zymogram, etc.

♣ **Session 5 : Characterization of α-amylase enzyme and determination the effect of physicochemical parameters on enzyme activity and stability,** provides an in-depth analysis of an amylolytic enzyme. This workshop will involve characterizing the enzyme's properties and investigating how various physicochemical parameters, such as temperature, pH, detergents, etc., affect its activity and stability?

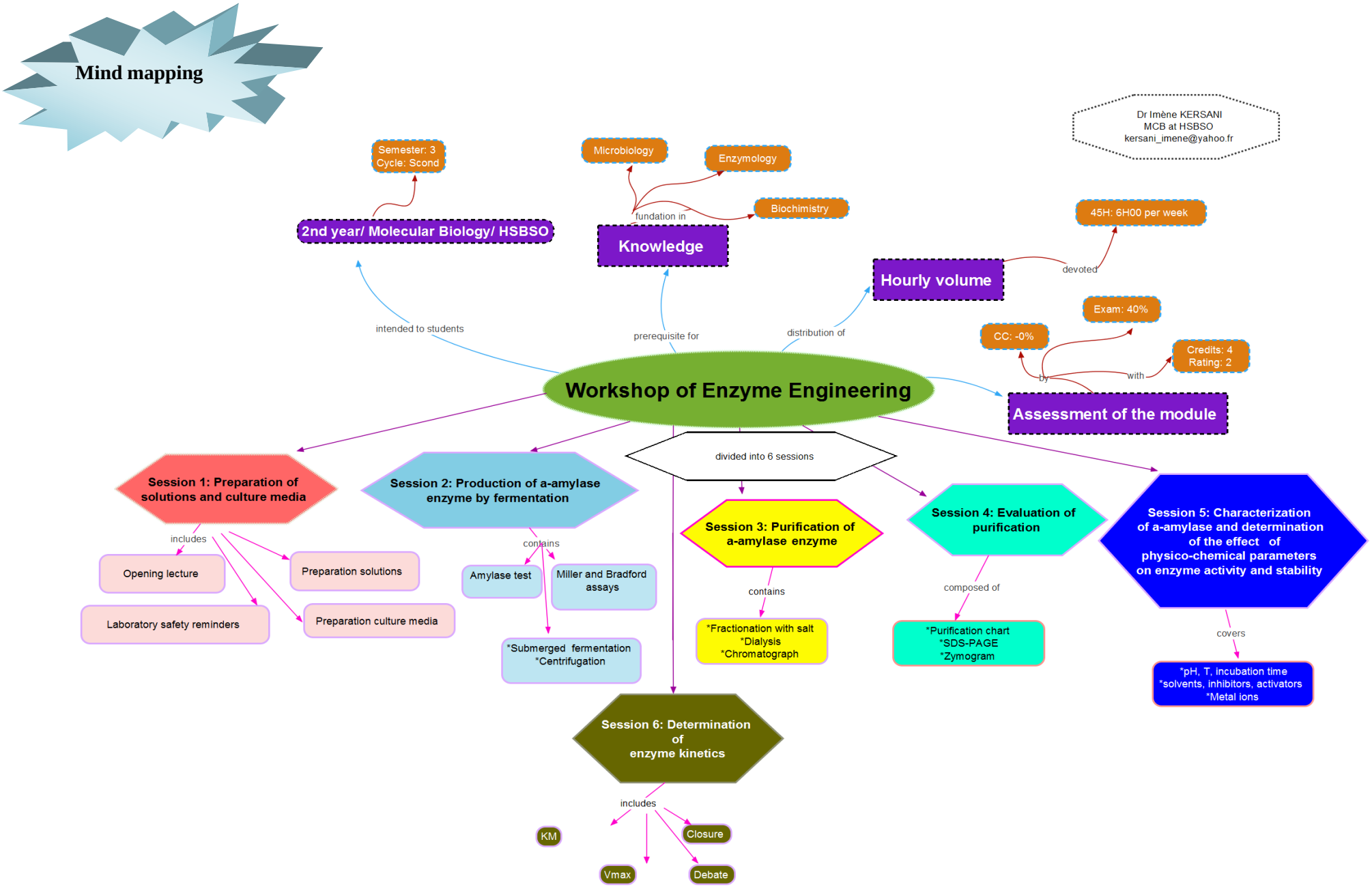
♣ **Session 6 : Determination of enzyme kinetics,** describes the transformation of substrate into product in the presence of the enzyme by determining the maximum rate of the enzymatic reaction (V_{max}) and the Michaelis constant (K_M). The session concludes with a discussion and closing remarks.

At the end of this workshop, students will be able to:

- Understand and apply safety measures in an educational laboratory.
- Master the process of isolating a target enzyme from a complex mixture using appropriate purification techniques.
- Optimize enzyme production conditions.
- Evaluate purification efficiency by compiling a purification table (chart) and utilizing electrophoresis techniques.
- Characterize the enzyme and determine the effects of physicochemical parameters on its activity and stability.
- Determine the kinetic parameters of the enzyme.

Knowledge and skills will be assessed through a continuous assessment (60%) and a final exam (40%). If competencies are not acquired, adapted learning methods, including remedial tests, will be proposed to address the individual needs of students.

Mind mapping



Mind mapping of the workshop of Enzyme Engineering.

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Abbreviations

$(NH_4)_2SO_4$: Ammonium sulfate

APS: Ammonium persulfate

B: Concentration of catalytic activity

BSA: Bovine serum albumin

$CaCl_2$: Calcium chloride

$Co(NO_3)_2$: Cobalt nitrate II

DMSO: Dimethyl sulfoxide

DNSA: 3,5-dinitrosalicylic acid

EDTA : Ethylenediaminetetraacetic acid

$FeCl_3$: Ferric trichloride or Iron (III) chloride

K_2HPO_4 : Dipotassium phosphate

KH_2PO_4 : Monopotassium phosphate

K_M : Michaelis constant

LB : Luria-Bertani media

$MgCl_2$: Magnesium dichloride

MW : Molecular weight

$NaHCO_3$: Sodium bicarbonate

$Pb(NO_3)_2$: Lead nitrate

R_f : Relative migration distance

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC: Size exclusion chromatography

SmF: Submerged fermentation

$SnCl_2$: Stannous chloride or Tin(II) chloride

TCA: Trichloroacetic acid

TEMED: N,N,N′,N′-tetramethylethylene-diamine

TRIS-HCl : Tris-(hydroxyméthyl)-aminométhane chlorhydrate

V_i : Initial rate

V_{max} : Maximum rate of the enzymatic reaction

Y: Purification yield

z: Enzyme activity

Z_{sp}: Specific activity

Workshop information

Institution : Higher School of Biological Sciences of Oran (HSBSO)

Department: Second Cycle

Public audience: Second Year

Speciality: Molecular Biology

Stream: Biological Sciences

Field: Natural and Life Sciences

Course title: Workshop of Enzyme Engineering (Atelier de Génie Enzymatique)

Teaching unit: Methodology 2

Credits: 04

Rating: 02

Semester : 03

Duration : 45h

Schedule: Monday 08h30-15h45

Laboratory: Enzyme Engineering

Responsable : Dr. I. KERSANI

Contact : via e-mail kersani_imene8@yahoo.com

Availability: *Common-room :* Thursday from 10:00 to 11:00

Response via email: The response will be provided within 24 hours of receiving the message, except in cases of unforeseen circumstances.

Introuction

Recently, there has been a growing demand for enzymes with improved activity, specificity, and stability, making enzyme engineering a vital tool for addressing the needs of various industrial processes. This engineered approach focuses on modifying the amino acid sequences of enzymes, which in turn affects their structural properties and, consequently, their enzymatic function while also creating new functionalities.

By employing appropriate techniques and strategies, engineered enzymes can be optimized for diverse industrial applications, significantly contributing to advancements in biotechnology and industrial efficiency.

To characterize and apply enzymes in various fields, including research and medicine, enzyme purification is essential. This process involves separating and isolating enzymes from cells, tissues, or entire organisms to obtain them in their purest form, maximizing their catalytic activity for further study or application.

The workshop begins with the preparation of culture media and solutions essential for producing the bacterial enzyme α-amylase via submerged fermentation, as well as for subsequent procedural steps. This is followed by a purification step aimed at isolating the enzyme from a crude extract of microbial cells, using various methods such as fractional precipitation with ammonium sulfate, dialysis, and chromatographic techniques like size exclusion chromatography. Finally, evaluating the purification process is considered essential for characterizing the enzyme and determining its associated physicochemical and kinetic parameters. In each step of the workshop, enzymatic activity is measured using the DNSA method, while total protein content is quantified using the **Bradford** method.

Workshop of enzyme engineering

Recommended prior knowledge *(outlined in the Foreword)*

A solid foundation in biochemistry, microbiology, and fundamental enzymology is essential to facilitate learning and ensure effective participation in the workshop.

Learning objectives *(outlined in the Foreword)*

At the end of this workshop, students will be able to:

- Understand and apply safety measures in a pedagogical laboratory.
- Master the process of isolating a target enzyme from a complex mixture using appropriate purification techniques.
- Optimize enzyme production conditions.
- Evaluate purification efficiency by compiling a purification table (chart) and utilizing electrophoresis techniques.
- Characterize the enzyme and determine the effects of physicochemical parameters on its activity and stability.
- Determine the kinetic parameters of the enzyme.

Soft skills

- Keep a laboratory notebook.
- Establish a roadmap.
- Learn the basics of scientific writing.
- Team-work.

Target skills

- Optimize enzyme production conditions.
- Capture the enzyme.
- Purify the enzyme by chromatography techniques.
- Establish the purification table.

Techniques

- Enzymatic assay by the DNSA method.
- Total protein assay by the **Bradford** method.
- Enzyme production via submerged fermentation (SmF).

- Fractional precipitation with ammonium sulfate.
- Salt removal by gel filtration/dialysis.
- Chromatography by gel filtration (size exclusion) and affinity chromatography.

Workshop program *(outlined in the Workshop schedule)*

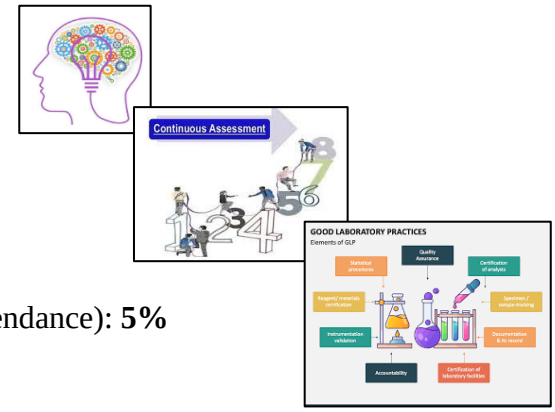
1. Opening lecture and overview/explanation. Safety reminders and laboratory guidelines.
2. Preparation of solutions and culture media.
3. Production of the α-amylase enzyme by fermentation.
4. Fractional precipitation of α-amylase with ammonium sulfate.
5. Removal of ammonium sulfate by dialysis.
6. Size-exclusion chromatography.
7. Affinity chromatography.
8. Evaluation of purification (Purification table, SDS-PAGE, Zymogram).
9. Characterization of α-amylase and determination the effect of physicochemical parameters on enzyme activity and stability.
10. Determination of enzyme kinetic parameters.
11. Evaluation, debate, and closure.

Workshop schedule

The workshop sessions will be held every Monday from 8:30 AM to 3:45 PM in the enzyme engineering laboratory. Group 2 will begin their classes during the first six sessions from October 7, 2024, to November 11, 2024. After that, there will be a rotation for the other group (G1), which will start the workshops until November 18, 2024, and continue until January 9, 2025. The workshop is planned as follows:

Week	Date	Group	Workshop Title
1	October 7, 2024	G2	**1. Preparation of solutions and culture media** * Opening lecture and explanation: Laboratory safety reminders
	November 18, 2024	G1	
2	October 14, 2024	G2	**2. Production of α-amylase enzyme by fermentation**
	November 25, 2024	G1	
3	October 21, 2024	G2	**3. Purification of α-amylase enzyme** * Fractional precipitation of α-amylase with ammonium sulfate * Removal of ammonium sulfate by dialysis * Size-exclusion chromatography * Affinity chromatography
	December 2, 2024	G1	
4	October 28, 2024	G2	**4. Evaluation of purification** * Purification chart * SDS-PAGE * Zymogram
	December 9, 2024	G1	
5	November 4, 2024	G2	**5. Characterization of α-amylase enzyme and determination the effect of physicochemical parameters on enzyme activity and stability**
	December 16, 2024	G1	
6	November 11, 2024	G2	**6. Determination of enzyme kinetics** ***** Evaluate, debate and closure
	January 6, 2025	G1	

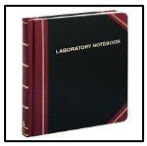
Continuous assessment and evaluation



- **Mode of continuous assessment:**
  - Tests: **25%**
  - Laboratory notebook: **30%**
  - Final workshop report: **40%**
  - Attendance (Good laboratory practices and attendance): **5%**

- **Mode of evaluation:**
  - Continuous assessment: **60%**
  - Exam: **40%**

Laboratory notebook



The laboratory notebook serves as a primary record of research. It is essential for documenting ideas, inventions, experimentation records, observations, and all relevant work details. Durable and customisable, it is a vital tool for researchers. Each student must bring their notebook daily, which should include the following:

- Full name, option, credits and rating.
- Objectives.
- Objective/purpose of experiment.
- Flow diagram.
- Step-by-step procedure, including all important test conditions/parameters.
- Supplementary information including citations.
- Observations and results (graphs, tables, figures, etc.).
- Raw data, calculated data, and any transcribed data.
- Conclusions: Whether the objective was achieved and/or suggestions for future experiments.

Final workshop report

The final workshop report is a comprehensive document containing the workshop objectives, methods and techniques, results, and overall insights from the workshop (IMRaD format). This report is essential for reflecting on the learning experience and assessing the effectiveness of the workshop activities.

The report should be well-organised, clearly written, and include all relevant data and analyses to provide a thorough overview of the workshop's findings and implications for future studies.

Session 1 : Preparation of solutions and culture media

- ♣ Opening lecture and explanation
- ♣ Laboratory safety reminders
- ♣ Preparation and storage of solutions and culture media

1.1 Introduction

In the enzyme engineering laboratory, a workshop will focus on enzymes, particularly bacterial enzymes, natural protein catalysts that accelerate chemical reactions within cells, thereby enhancing the efficiency of processes in living organisms.

The workshop will start with an opening lecture outlining the main objectives and expectations for laboratory work. It will cover the handling of microorganisms and chemical agents, which pose hazards to manipulators and the environment. Strict adherence to laboratory safety rules is essential to ensure a secure environment and maintain the integrity of experiments throughout all workshop sessions.

1.2 Objectives

The workshop aims to achieve the following objectives:

- Follow the general laboratory safety instructions.
- Follow correct procedures for preparing solutions and culture media used during the workshop sessions.

1.3 Laboratory safety rules and guidelines

Ensuring safety in the laboratory is essential to protect both personnel and the environment. Students must strictly adhere to the following guidelines to create a secure working atmosphere and minimize risks during laboratory activities:

1. Do not eat, drink, smoke or store food in the laboratory, even temporarily.
2. It is mandatory to wear a long-sleeved, buttoned blouse, made of 100% cotton and non-flammable.
3. Familiarize yourself with the locations of fire extinguishers and safety equipment in the laboratory.
4. Long hair must be tied back during handling to prevent accidental burns or contamination. Open-toed shoes (such as sandals or flip-flops) are unacceptable in the laboratory.
5. Wash hands thoroughly before and after handling, as well as before leaving the laboratory, even temporarily.
6. Clean the benchtop with bleach (Javel water) before and after handling.

7. Exercise caution when using Bunsen burners, ensuring that flammable liquids, including alcohol, and combustible materials, such as paper, are kept away from the open flame.
8. Handle all living cultures of microorganisms (bacteria, yeast, etc.) as potential pathogens. Avoid spills and contamination, and notify your instructor immediately if a spill occurs.
9. When returning to the laboratory, all personal belongings and items (documentation, coats, backpacks, etc.) must be left in the designated area and must not be kept on the laboratory benchtop.
10. Carefully read the entire procedure before beginning any laboratory experiment to avoid mistakes that could compromise your safety.
11. Avoid opening windows during handling.

1.4 Preparation of solutions and culture media

1.4.1 Products and equipment

All items needed for this session, including products, equipment, and instruments, are shown in the **table** below:

Table 1.1 : Products and equipment for session 1.

Products	Equipment/ instruments
• Distilled water • NaOH • Coomassie Blue G-250 • Ethanol (95%), • Maltose • Luria-Bertani media (LB liquid and solid) • Starch • Potassium phosphate buffer • Orthophosphoric acid (85%) • Potassium sodium tartrate tetrahydrate • DNSA • Urea	• pH meter • Precision balance • Refrigerator • Magnetic stirrer with stirring bar • Test tubes • Petri plates • Spatulas • Inoculation loops platinum • Erlenmeyer flasks • Whatman filter paper • Alcohol wash bottle • Bunsen burner • Vortex • Pipette tips, • Micropipettes • Beakers • Autoclave • Incubators • Graduated cylinder • Filter funnel • Watch glass • Dark bottles • Fume hood/ chemistry hood

1.4.2 Amount of culture media, buffer and reagents

The content of the solutions, culture media and reagents are represented in the following **table**. For media containing agar (solid), it is important to heat the mixture to dissolve the agar before autoclaving. Additionally, all culture media (both liquid and solid) must be sterilized in an autoclave at 121°C for 20 min, and the pH of growth media must be adjusted before sterilization.

It should be noted that the solutions are stored under specific conditions for later use. Culture media are kept in the refrigerator at 4°C, while reagents are stored in dark glass bottles away from heat and light.

Table 1.2 : Amount of solutions and culture media.

Solutions		**Culture media**	
Compound	**Amount**	**Compound**	**Amount**
DNSA reagent (200mL) • DNSA (3,5-dinitrosalicylic acid) • 200 mL of NaOH (2N) • Potassium sodium tartrate tetrahydrate • Distilled water	 10g 16g 300g 1L	**LB broth** (200 mL) • Peptone • Yeast extract • Sodium chloride • Distilled water pH 7	 10g 5g 5g 1L
Potassium phosphate buffer, 50 mM, pH 7 (4L) • Monopotassium phosphate (KH_2PO_4) • Dipotassium phosphate (K_2HPO_4) • Distilled water	 3.154g 4.672g 1L	**LB agar media: + 1% starch** (100 mL) • Peptone • Yeast extract • Sodium chloride • Agar agar • Distilled water pH 7	 10g 5g 5g 15g 1L
Buffered starch, 1% (100mL) • Starch • Potassium phosphate buffer, 50 mM, pH 7	 1g 100Ml	**Optimized enzyme production media: Starch broth** (100 mL) • Strach • Tryptone pH 7	 1% 0. 5%
Bradford reagent (300mL) • Coomassie Blue G-250 • Ethanol 95% • Orthophosphoric acid 85% • Distilled water	 5mg 2.5mL 5mL 50 mL		
NaOH 2N **HCl 4N**	200 mL 100mL		

1.5 Conclusion

At the end of the session, each student will be able to prepare solutions and culture media while respecting laboratory safety procedures. The prepared solutions and culture media will be stored under specific conditions for later use.

1.6 Exercise

1. What steps would you follow to prepare 250 mL of a 2N HCl solution ? Refer to the pictogram for detailed guidance on the necessary calculations.

2. To prepare 1 L of a 3 mM potassium phosphate buffer at pH 6, how much monopotassium phosphate (KH_2PO_4) and dipotassium phosphate (K_2HPO_4) should be added, using the following resource: *AAT Bioquest buffer preparation guide*.

1.7 Solution

1. Preparation of 250 mL of HCl (2N) :

a) Determine the concentration of HCl

$C=\frac{\rho.d.\%}{M}$= 12.06 mol/l

b) Calculate the volume of concentrated HCl needed:

$C_1V_1=C_2V_2$==> V_1=41.49 mL

c) Measure 41.49 mL of concentrated HCl.

d) Dilute with distilled water:

Add distilled water to the concentrated HCl in a 250 mL volumetric flask.

Make sure to add water up to the 250 mL mark, ensuring accurate volume.

e) Mix thoroughly:

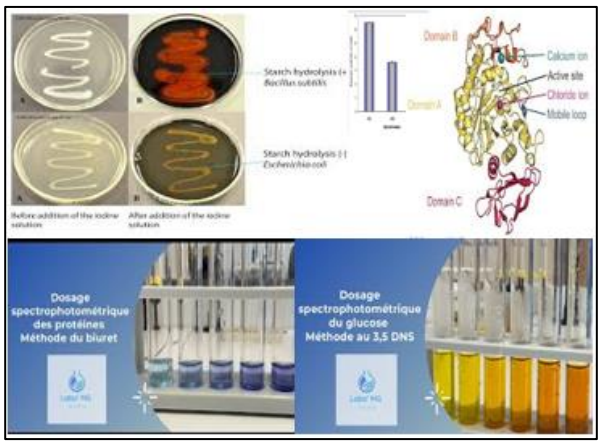
Cap the volumetric flask and invert it several times to mix the solution evenly.

2. Preaparation of 1 L of potassium phosphate buffer (3mM, pH 6) :

K_2HPO_4=72.151 mg

KH_2PO_4=351.159 mg

Session 2 : Production of α-amylase enzyme by fermentation



- ♣ Starch hydrolysis test
- ♣ α-amylase enzyme production by submerged fermentation
- ♣ Enzyme recovery
- ♣ α-amylase activity and total protein content

2.1 Introduction

The increasing demand in industrial and biotechnological sectors for more efficient enzymes with improved biochemical properties, particularly thermostability and thermotolerance, calls for a prompt and timely response. Among the enzymes showing significant promise, α-amylase (EC 3.2.1.1) stands out as one of the most important industrial endoamylases. It catalyzes the hydrolysis of internal α-1,4 glycosidic bonds, producing glucose, maltose, and dextrins.

Widely distributed in nature, α-amylase can be sourced from plants, animals, and microorganisms, with microbial sources dominating industrial applications, particularly in fermentation, starch saccharification, analytical chemistry, and ethanol production for fuel.

2.2 Objectives

The workshop aims to achieve the following objectives:

- Determine the ability of microorganisms to hydrolyze starch.
- Produce α-amylase enzyme by submerged fermentation (SmF).
- Recover the enzyme by centrifugation.
- Determine the amylolytic activity using the **Miller** method.
- Quantify protein content using the **Bradford** method.

2.3 Products and equipment

All necessary items for this session are listed in the **table** below:

Table 2.1 : Products and equipment for session 2.

Products	Equipment/ instruments
➢ Biological strain: *Bacillus subtilis* TL03. ➢ Products: • Distilled water • Lugol • Iodine crystals • LB media (liquid and solid) • Starch, soluble, potato • DNSA reagent • **Bradford** reagent • BSA	• Petri plates • Erlenmeyer flasks • Beakers • Spatulas • Bunsen burner • Pipette tips • Micropipettes • Incubators/shaking incubators • Centrifuge • Inoculation loops platinum • Test tubes

• Maltose • Ice cubes	• Water bath • Vortex • Spectrophotometer • Ice tray • Falcon conical tubes • Fume hood/ chemistry hood

2.4 Experimental protocols

The present work revolves around the study of the α-amylase enzyme produced by the bacterial strain *Bacillus subtilis* TL03.

Bacillus species are rod-shaped, catalase-positive, endospore-forming, aerobic or facultatively anaerobic, Gram-positive bacteria. In some species, cultures may turn Gram-negative with age. These bacteria are ubiquitous in nature and can tolerate extreme environmental conditions. *Bacillus* species are utilized in numerous medical, pharmaceutical, agricultural, and industrial processes, due to their wide range of physiologic characteristics and their ability to produce enzymes, antibiotics, and other metabolites.

One of the most important sources of industrial α-amylase is thermophilic *Bacillus* sp. Several strains, including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Bacillus amyloliquefaciens*, and *Bacillus cereus*, exhibit this amylolytic activity, efficiently hydrolyzing starch into shorter oligosaccharides. These strains are extensively used in industrial applications due to their capability to produce significant levels of α-amylase under diverse environmental conditions, making them highly valuable in sectors such as food processing and fermentation.

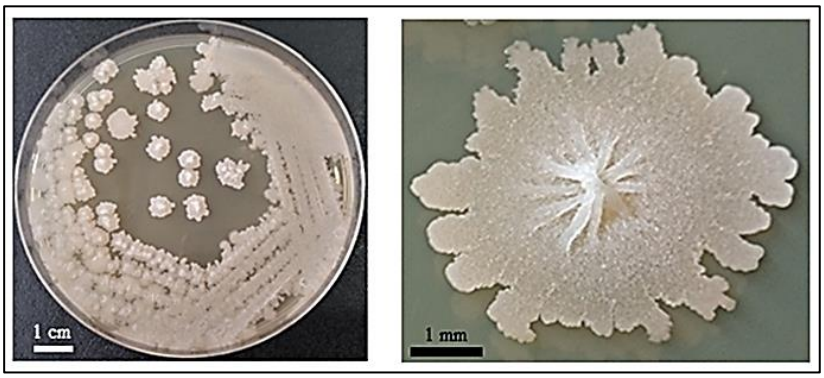


Figure 2.1: Growth of *Bacillus subtilis* on LB agar media.

2.4.1 Starch hydrolysis test (amylase test)

2.4.1.1 Principle

The starch hydrolysis test, also known as the amylase test, is a biochemical assay used to determine the ability of bacteria to produce amylase and utilize starch as a carbon source. Bacteria capable of producing extracellular amylase enzymes can catalyze the polysaccharide into shorter oligosaccharides by cleavage of the α-D-(1-4) glycosidic bonds in the inner part of the amylopectin or amylose chains.

Starch itself is composed of two components: amylose, a linear polysaccharide, and amylopectin, a branched polysaccharide (**Figure 2.2**). To evaluate starch degradation, starch agar is used as a nutrient medium supplemented with 1% starch. This agar is particularly effective for demonstrating the degradation of starch by specific bacterial species, notably those within the *Bacillus* genus.

Upon the addition of iodine solution, the hydrolysis of starch can be identified by observing the formation of a clear halo around the bacterial growth in the inoculated and incubated starch medium (**Figure 2.3**). This clear zone indicates successful starch hydrolysis and confirms the presence of amylase-producing bacteria.

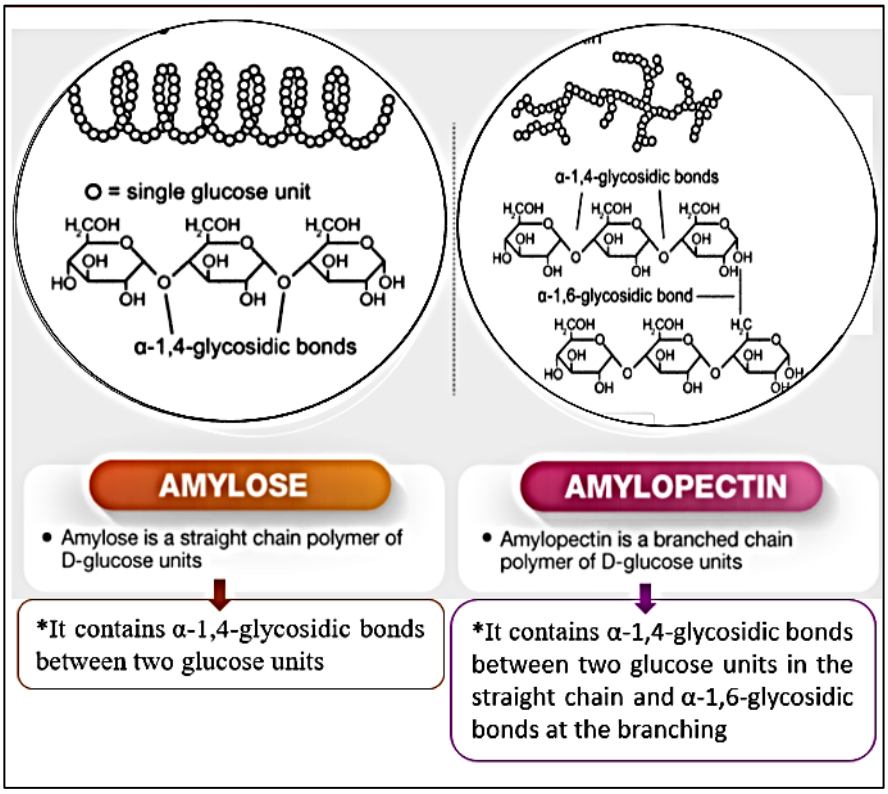


Figure 2.2: Starch structure.

2.4.1.2 Procedure

1. Aseptically, make a single-line streak inoculation of microorganism to be tested on LB starch plate.
2. Incubate bacterial culture at 50°C for 24 h.
3. After incubation, add iodine crystals or Lugol's solution to the surface of the plates using one of the following methods:
    - **1st method:** Expose the bacterial colonies on starch plates to iodine vapor from sublimating crystals in a beaker for 30 min.
    - **2nd method:** Flood the surface of the plates with Lugol's solution for 30 s.
4. Observe the plates for a clear zone around the bacterial growth line, indicating starch hydrolysis.

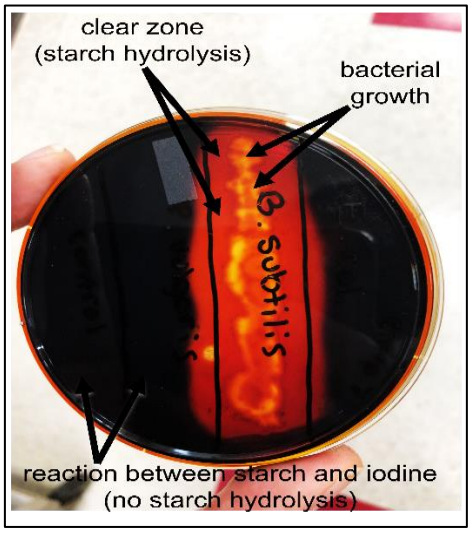


Figure 2.3: Positive test of starch hydrolysis.

2.4.1.3 Reading

In the starch hydrolysis test, if the inoculated bacteria produce amylase, the surrounding starch is hydrolyzed, and no dark blue coloration appears when iodine is added. Therefore, a clear zone forms around the colony, indicating successful hydrolysis. Conversely, if the bacteria do not produce amylase, the starch remains intact, and the addition of iodine results in a dark blue coloration, indicating the absence of hydrolysis.

2.4.2 Production of α-amylase by submerged fermentation (SmF)

2.4.2.1 Principle

Submerged fermentation involves cultivating microorganisms in a liquid medium, where the biomass is suspended or immersed. In this process, various nutrients are either dissolved or

present as particulate solids, allowing for effective homogenization of the medium's components to optimize growth conditions.

2.4.2.2 Procedure

1. Perform an 18h pre-culture of *Bacillus subtilis* in LB broth.
2. Inoculate two liquid starch media, (LB+ 1% starch and optimized amylase production broth) with 4% of the pre-culture adjusted to 0.5 McFarland, in Erlenmeyer flasks.
3. Incubate the cultures overnight in a shaking incubator at 50°C and 100 rpm.

2.4.2.3 Reading

The appearance of turbidity indicates bacterial growth resulting from the hydrolysis of starch by α-amylase. This cloudiness is due to the breakdown of starch into smaller, soluble sugars, which supports microbial growth.

2.4.3 Recovery of α-amylase by centrifugation (*extraction of α-amylase enzyme****)***

2.4.3.1 Principle

Centrifugation is a mechanical process that employs centrifugal force to separate mixtures of particles with varying masses or densities suspended in a liquid medium. When a vessel (tube or bottle) containing a mixture of proteins or other particulate matter (e.g., bacterial cells) is rotated at high speeds, the inertia of each particle generates an outward-directed force proportional to its mass. This outward movement is countered by the resistance of the liquid, which affects the particles' motion. As a result, larger and denser particles move outward more rapidly than smaller or less dense ones. Consequently, when suspensions are centrifuged, a pellet forms at the bottom of the vessel, enriched with the most massive particles, while lighter particles remain suspended in the supernatant.

2.4.3.2 Procedure

1. After incubation under appropriate conditions, centrifuge the culture at 5000 rpm for 20 min at 4°C.
2. Carefully collect or recover the supernatant.
3. Store the supernatant at -20°C for future use.

2.4.4 Amylolytic activity assay

2.4.4.1 Principle

The α-amylase activity is measured using a colorimetric method of **Miller (1959)** with 3,5-dinitrosalicylic acid (DNSA) reagent. In this method, starch is hydrolyzed by α-amylase into maltose, which is quantified by the reduction of DNSA. This process detects the presence of

free carbonyl group (C=O) in reducing sugars, involving the oxidation of aldehyde functional group (−CH=O) and ketone functional group (R−C(=O)−R'). During the reaction, DNSA is reduced to 3- amino-5-nitrosalicylic acid (ANSA). Under alkaline conditions and heat, DNSA is converted into a reddish-orange complex that exhibits maximum absorbance at 540 nm.

Figure 2.4: Reducing sugar by DNSA method.

2.4.4.2 Procedure & Reading

a) Calibration/Standard range

1. Prepare maltose buffer at a concentration of 2 mg/mL by dissolving 2 g of maltose in 1 L of potassium phosphate buffer, 50 mM pH 7.
2. Prepare maltose standards with concentrations ranging from 0 to 2 mg/mL, making up a final volume of 1 mL for each.
3. Mix 1V of each standard with 1V of DNSA reagent.
4. Place the standards at boiling water bath for 5 min.
5. Read absorbance at 540 nm.
6. Construct a standard curve using the absorbance values from the serial dilutions of maltose.

b) Enzymatic assay of α-amylase (+ effect of incubation time on α-amylase production**)**

1. Preheat 1% buffered starch to 70°C.
2. Mix 1V of supernatant with 1V of buffered starch (V/V).
3. Incubate the tubes at 70°C, taking samples every 5 min (from t5 to t30). For tube 0 (t0), do not incubate.
4. Stop the reaction by adding 2V of DNSA to each tube.
7. Incubate the samples in water bath at 100°C for 5 min or 98°C for 10 min.
5. Measure the amount of reduced DNSA at 540 nm.
6. Determine the maltose concentration (mg/mL) through the calibration curve.

7. Calculate the enzyme activity (z) in international units (IU), corresponding to the enzyme's ability to catalyze the transformation of 1 µM of substrate per minute, using the following equation:

$$z\ (IU) = \frac{(maltose\ concentration).(total\ volume\ in\ mL)}{reaction\ time\ in\ min} = \frac{d[P]}{dt} . V = \frac{number\ of\ moles\ (n)}{t}$$

2.4.5 Protein assay

2.4.5.1 Principle

The Bradford protein assay is a widely used method for quantifying protein content in crude enzyme preparations, as established by **Bradford (1976)**. This colorimetric method relies on the interaction between the side-chain chemical groups of various amino acids and the Coomassie Brilliant Blue reagent (G250), resulting in a purple-blue color. The absorbance of this color is measured at a wavelength of 595 nm, providing a quantitative assessment of protein content. Bovine Serum Albumin (BSA) is used as the standard.

2.4.5.2 Procedure & Reading

a) Calibration/Standard range

1. Prepare 5 mL of a 1 mg/mL aqueous solution of BSA.
2. Establish a standard range containing between 0 µg and 120 µg of BSA.

b) Enzym assay

1. Mix 20 µL of the sample to be tested with 980 µl of potassium phosphate buffer (50mM, pH7).
2. Add 1 mL Bradford reagent to the mixture (sample and standards).
3. Incubate the mixture at room temperature in a dark place for 15-30 min.
4. Measure the absorbance at 595 nm.
5. Create a standard curve by graphing the following data (Absorbance vs. protein quantity).

2.5 Conclusion

At the end of the workshop, each student will be able to produce the α-amylase enzyme through submerged fermentation in starch broth. They will learn to qualify this production using the starch hydrolysis test and quantify it by measuring the concentrations of reducing sugars and the total protein content after centrifugation and recovery of the supernatant.

2.6 Exercise

The catalytic activity of enzymes can be quantified by measuring the changes in concentration of substrates or products during the reaction.

The study of this activity is carried out on enzyme X, which has a concentration of 10 g/L and is present in a 5 mL reaction mixture. This enzyme converts substrate S into product P in 10 min.

1. Define the enzyme activity.
2. What are the key elements that affect the outcome of an enzymatic assay?
3. Calculate the catalytic activity of enzyme X in IU.

Data : Molar mass of substrate S is 342.297 g/mol.

2.7 Solution

1. Enzyme activity is the measure of the functional efficiency of enzymes in biological systems. It corresponds to the content of enzyme required to catalyze the transformation of 1 micromole (1 μmol) of substrate per minuteunder specific conditions. This is expressed in ″IU″.

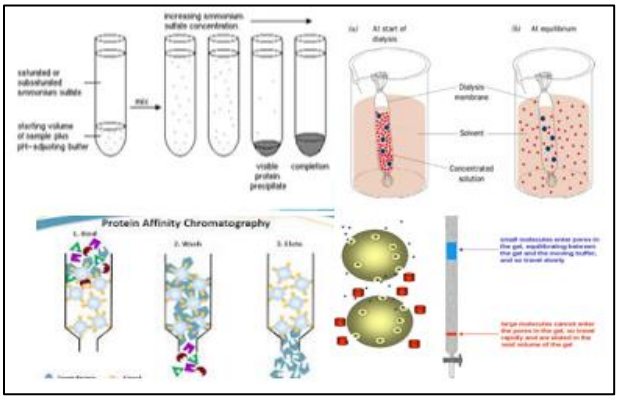
2. Most effective factors on an enzymatic assay:

pH, temperature, enzyme concentration, substrate concentration, and reaction time.

3. Catalytic activity of enzyme X in IU :

$$z = \frac{n}{t} = 15 \text{ IU}$$

Session 3 : Purification of α-amylase enzyme



- ♣ Fractionation with ammonium sulfate
- ♣ Dialysis
- ♣ Chromatography techniques

3.1 Introduction

Enzyme purification is a process of separating and isolating enzymes from other cellular components to obtain pure enzymes with the highest catalytic activity. Thousands of proteins have been successfully purified based on characteristics such as solubility, size, charge, and specific binding affinity.

Several methods exist for enzyme purification, each tailored to specific properties of the target enzyme. For instance, fractional ammonium sulfate precipitation is commonly used for protein precipitation but may interfere with downstream applications like SDS-PAGE. Therefore, it is essential to eliminate contaminants, including salts, through methods like dialysis or chromatography, which enables rapid and simple separation of molecules soluble in water or certain organic solvents based on their size or molar mass.

3.2 Objectives

The workshop aims to achieve the following objectives:

- Purify the α-amylase enzyme through fractional precipitation with ammonium sulfate.
- Remove the salt from the enzyme using dialysis.
- Analyse the purification of the enzyme via chromatography.

3.3 Products and equipment

All required items for this session are presented in the **table** below:

Table 3.1 : Consumable and non-consumable equipment for session 3.

Products	Equipment/ instruments
➢ <u>Enzyme sample:</u> Supernatant of α-amylase enzyme ➢ <u>Products:</u> • Ammonium sulfate $(NH_4)_2SO_4$ powder • Potassium phosphate buffer (50Mm, pH7) • Sodium bicarbonate ($NaHCO_3$) • Ethanol	• Refrigerated centrifuge • Test tubes • Ice tray • Falcon conical tubes • Refrigerator • Beakers • Spatulas • Dialysis tubing/ dialysis bag (6.4mLx28.6mm) • Magnetic stirrer with stirring bar

• EDTA • Distilled water • Sephadex G75 powder	• Vortex • Water bath • Fume hood/ chemistry hood • Cotton • Column (1cmx70cm LxD) • Precision balance • pH meter

3.4 Experimental protocols

3.4.1 Fractional precipitation of α-amylase by ammonium sulfate (salting-out technique)

3.4.1.1 Principle

Salting out is a purification method that exploits the reduced solubility of certain molecules in solutions with very high ionic strength. The general mechanism of fractional precipitation relies on salts and solvents that decrease or reduce the amount of 'free' water in the system. This reduction prevents proteins from forming hydrogen bonds with water, allowing the salt to facilitate interactions between the proteins, leading to the formation of aggregates that subsequently precipitate out of the solution.

Ammonium sulfate is an ideal salt for enzyme fractionation due to its ability to maintain the protein's structure and activity. It is highly soluble, inexpensive, exhibits low toxicity to enzymes, and serves as a preservative.

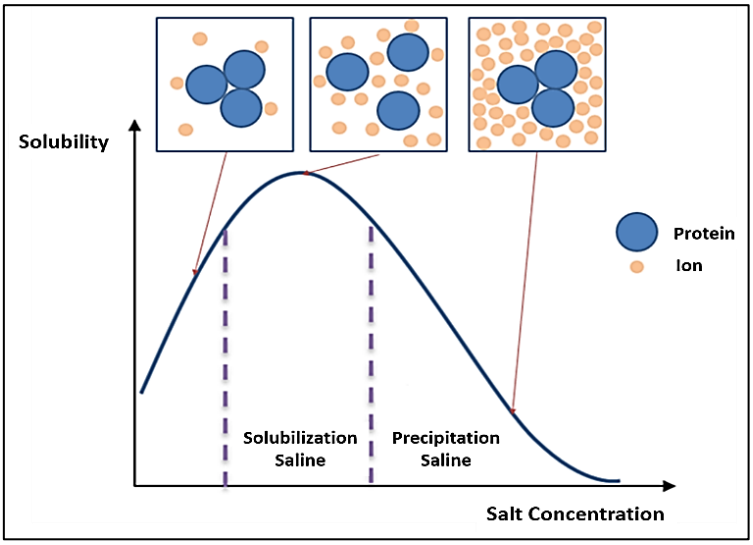


Figure 3.1: Salting in and salting out.

3.4.1.2 Procedure

The α-amylase, produced by submerged fermentation and recovered by centrifugation, was precipitated by different concentrations of ammonium sulfate.

1. Prepare a saturated solution of ammonium sulfate (20%).
2. Gradually add the specified quantity of solid ammonium sulfate to the supernatant using an ice tray, while gently agitating. Ensure the solid ammonium sulfate is dried in an incubator at 80°C beforehand. Heat gently to dissolve all the ammonium sulfate, then cool to working temperature on a magnetic stirrer. The formation of ammonium sulfate crystals indicates that the solution is fully saturated.
3. After incubation for 18h at 4°C with gentle agitation, centrifuge the precipitate at 4500 rpm for 30 min at 4°C. Resuspend the pellet in 50 mL of potassium phosphate buffer (50mM, pH 7), corresponding to 1/10 of the initial volume of the supernatant used.
4. Add the appropriate volume of saturated ammonium sulfate or solid ammonium sulfate to the sample to get the desired concentration, ranging from 30% to 100%, using the online website: http://encorbio.com/protocols/AM-SO4.htm or the table below.
5. Repeat the same procedure for each saturation.

Table 3.2 : Amount of ammonium sulfate needed to reach the desired saturation level in a 10 mL sample at 4°C.

Desired saturation [%]	**Amount (g)**	**Final volume (mL)**
5	0.26	10.14
10	0.53	10.28
20	1.10	10.58
30	1.70	10.90
40	2.33	11.24
50	3.01	11.60
60	3.73	11.98
70	4.50	12.39
80	5.33	12.83
90	6.21	13.30
100	7.17	13.81

3.4.2 Salt removal by dialysis

3.4.2.1 Principle

Enzymes can be separated from small molecules, such as salts, by dialysis through a semi-permeable membrane, like a cellulose membrane with pores. Desalting by dialysis is a common step in protein purification, particularly for enzymes following fractional precipitation with ammonium sulfate or other salt-based methods. This technique effectively removes salts and non-diffusible impurities from the sample by allowing small diffusible molecules to exit the dialysis bag into the surrounding buffer, while retaining the protein or enzyme of interest. The movement of salt molecules will cease once equilibrium is reached, at which point the buffer is changed to facilitate continued diffusion and salt removal.

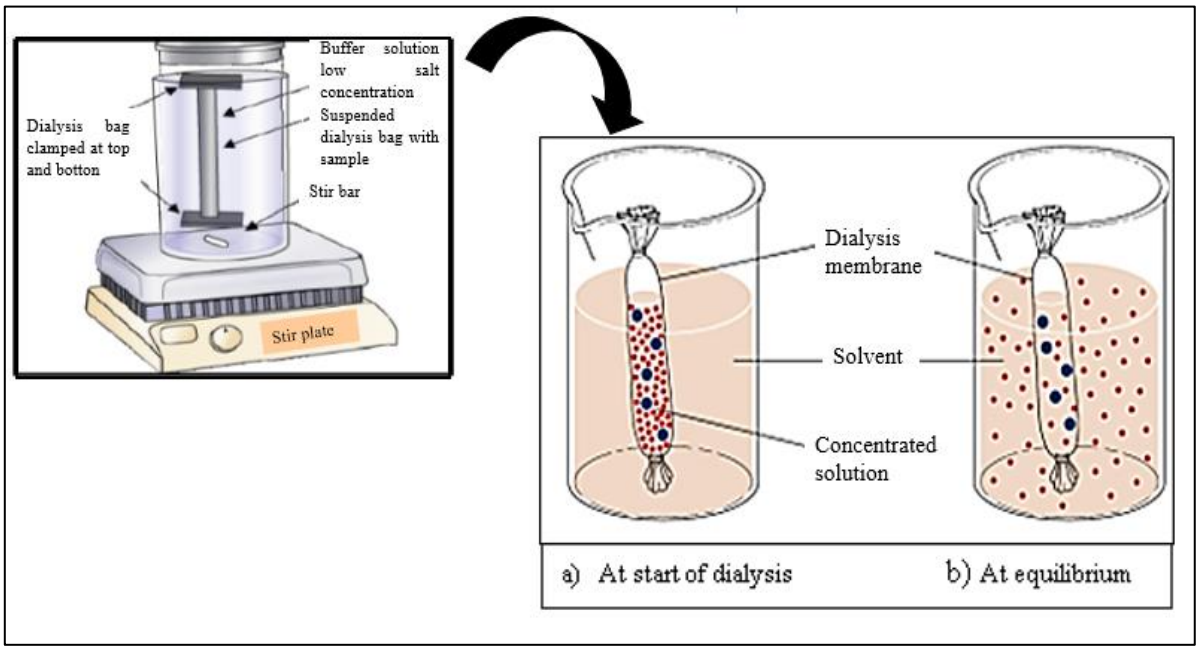


Figure 3.2: Principle of dialysis.

a) Only small molecules can diffuse through the pores of the dialysis bag/tubing, which is shown here as a tube knotted at both ends,

b) At equilibrium, the concentrations of small molecules are nearly identical inside and outside the bag, whereas the macromolecules remain in the bag.

3.4.2.2 Procedure

a) Preparation of the dialysis tubing

1. Cut off the dialysis tubing (Spectra/POr Member MWCO 6-8000: volume=6.4mL, diameter of the dry cylinder: 28.6mm), incubate it in distilled water for 10 min, then in ethanol (50 %) for 10 min.
2. Soak the dialysis tubing in a boiling solution of 2% sodium bicarbonate and 1 mM EDTA.
3. Store it in ethanol (50%) at 4°C.

4. Before use, rinse the dialysis tubing with distilled water, followed by phosphate buffer (50mM, pH 7) on both the outside and inside.

b) Dialysis

1. Introduce the supernatant in a dialysis tubing/bag.
2. Seal the tubing, using standard dialysis closures to prevent leaks.
3. Place the sealed tubing in a beaker with gentle shaking to enhance salt removal, and maintain it at 4°C overnight in 1 L of phosphate buffer (dialysis buffer: 50 mM, pH 7) until α-amylase activity is stabilized.
4. Change the dialysis buffer at least once during the equilibrium period.
5. Transfer supernatant to a clean tube.

3.4.3 Purification of α-amylase by size exclusion chromatography (SEC: gel filtration)

3.4.3.1 Principle

Removal of ammonium sulfate can be achieved using various purification techniques, such as gel filtration chromatography with Sephadex G25. In this process, ammonium sulfate, a low molecular weight salt, is effectively separated from larger molecules, like proteins or enzymes, through a porous gel made of spherical beads with a specific size distribution of pores. Separation occurs as molecules of differing sizes are either included in or excluded from these pores, depending on their size and the time required to pass through the column. Consequently, high molecular weight molecules elute first, while the smaller salt molecules elute later.

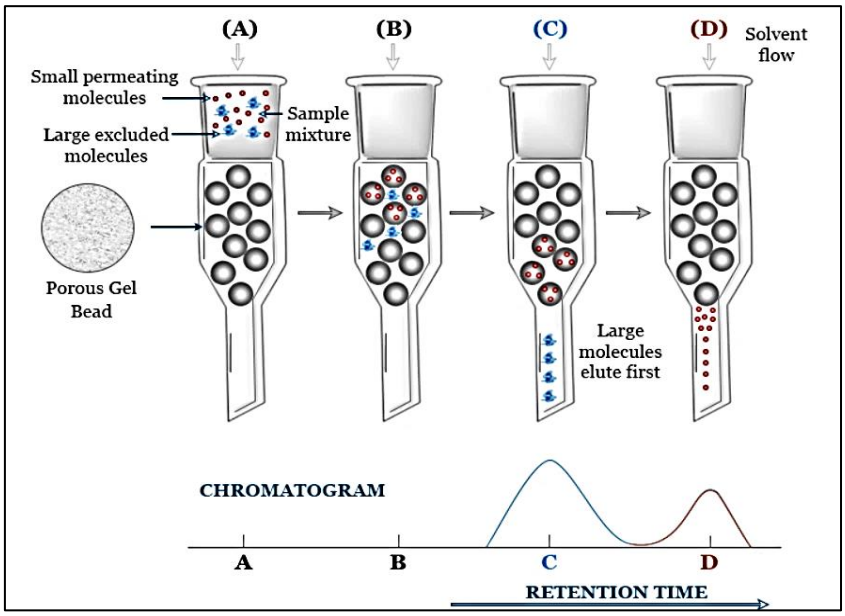


Figure 3.3: Principle of size exclusion chromatography.

3.4.3.2 Procedure

a) Preparation of the chromatography column

1. Rinse the chromatography column (1cm x 70 cm Lx D) with distilled water.
2. Place a small piece of cotton at the bottom of the column to control the flow, and then assemble the column vertically.
3. Weigh out 3.846 g of Sephadex G75 (each 1 g of Sephadex prepares 12 to 15mL of gel).
4. Add an excess of potassium phosphate buffer (50 mM, pH7, approximately 300 mL).
5. Incubate the mixture in a water bath at 90°C for 3 h.
6. After swelling, stir the gel carefully using a glass rod to avoid breaking the gel beads.
7. Gradually pour the gel into the column until gelling and complete stabilization occur, filling up to 45 mL. Prevent the gel bed from drying by continuously adding buffer.

b) Chromatography process

1. After the gel has gelled and stabilized, gradually introduce 5 mL of the precipitated protein sample (dialysate) along the walls of the column.
2. Add potassium phosphate buffer to the column (50 mM, pH 7).
3. Collect α-amylase enzyme fractions (1mL) with an elution rate of 1 mL/40 s. The entire sample will probably be eluted after about 1 h of separation.
4. Measure the enzymatic activity of each fraction and determine the total protein content.
5. Combine the enzymatically active fractions into a single tube and store them at -20°C.

At the end of the purification, sum up the results of the final purified sample, including volume, total proteins, amylase activity, specific activity, and overall purification yield compared to the crude sample. The purification yield (Y) can be calculated using the formula below.

$$Y = \frac{total\ enzymatic\ activity\ recovered\ during\ the\ purification\ step\ (IU)}{total\ initial\ enzyme\ activity\ (IU)} \text{ x } 100\%$$

Remark:

After the purification methods, the specific activity of an enzyme (Z_{sp}) should be determined, using the **Miller** and the **Bradford** assays (Session 2: Production of α-amylase enzyme by fermentation). It is expressed as the number of units per milligram of protein. This activity is calculated using the following equation:

$$Z\ sp\ \left(\frac{IU}{mg}\right) = \frac{B}{C} = \frac{enzyme\ activity\ (IU)}{proteins\ quantity\ (mg)} = \frac{z}{mg\ of\ proteins}$$

Where $B=\frac{z}{V}$,with B being the concentration of catalytic activity (IU/mL)

3.4.4 Purification of α-amylase by affinity chromatography

3.4.4.1 Principle

Affinity chromatography is a powerful and versatile technique in chemistry that exploits the specific binding properties of biomolecules to separate them from complex mixtures. It is one of the most widely used chromatographic methods for purifying a specific molecule or group of molecules. This method is based on highly specific biological interactions between two molecules, such as enzyme-substrate or receptor-ligand interactions. These typically reversible interactions are used for purification by immobilizing one of the interacting molecules, known as the affinity ligand, onto a solid matrix to form the stationary phase, while the target molecule is in the mobile phase.

The target molecule can be eluted from the column by altering the conditions of the mobile phase, such as changing the pH, increasing the salt concentration, or using an organic solvent. These changes disrupt the specific interactions between the target molecule and the affinity ligand, allowing the purified molecule to be released from the stationary phase and collected in the eluent.

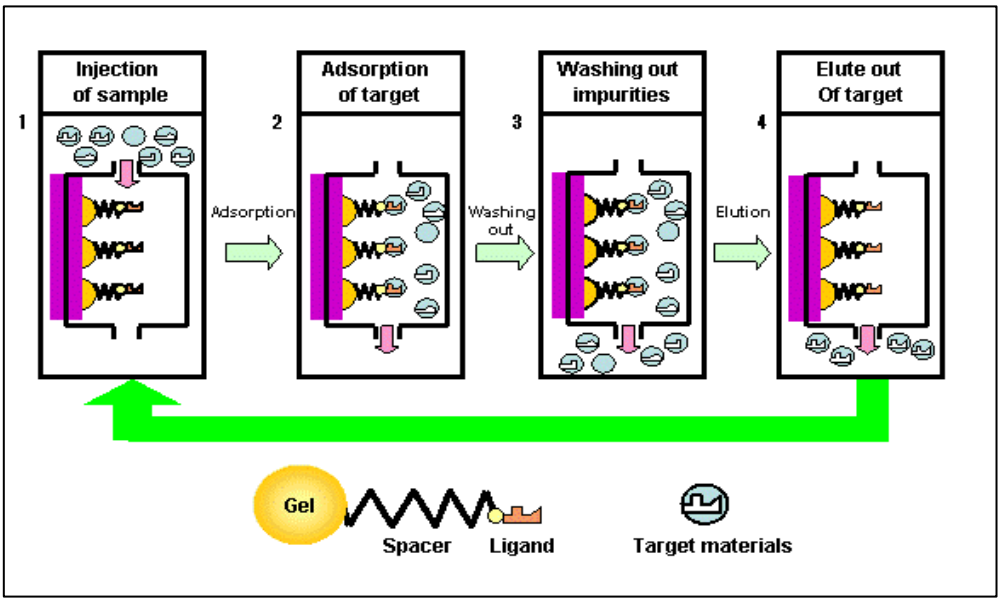


Figure 3.4: Principle of affinity chromatography.

3.4.4.2 Procedure

a) Affinity chromatography process steps

1. Prepare the affinity column by attaching the immobilized ligand specific to α-amylase.
2. Load the protein mixture containing α-amylase onto the column.
3. Bind the proteins to the ligand.

4. Wash the column with binding buffer to remove non-specific proteins and unwanted materials.
5. Wash off the loosely bound proteins.
6. Elute the tightly bound α-amylase by altering the mobile phase conditions and collect the eluted fractions.
7. Measure the protein concentration and enzymatic activity in the collected fractions.
8. Regenerate the column for future use.

3.5 Conclusion

At the end of the session, each student will be able to purify enzymes through fractionation with ammonium sulfate, followed by dialysis and chromatography techniques based on size and affinity. Students will also gain hands-on experience in understanding the principles behind each purification method.

3.6 Exercise

Choose the correct answer(s):

1. Size-exclusion chromatography
   - □ Planar chromatography
   - □ Column chromatography
   - □ Stationary phase contains a porous mesh of inert spherical particles
   - □ Mobile phase contains buffer solution

2. The amylolytic enzyme α-amylase
   - □ *Bacillus subtilis*
   - □ Exoglycosidase
   - □ Randomly cleaves the 1,4-α-D-glycosidic linkages between adjacent glucose units in the amylose chain
   - □ Randomly cleaves the 1,6-α-D-glycosidic linkages between adjacent glucose units in the amylose chain

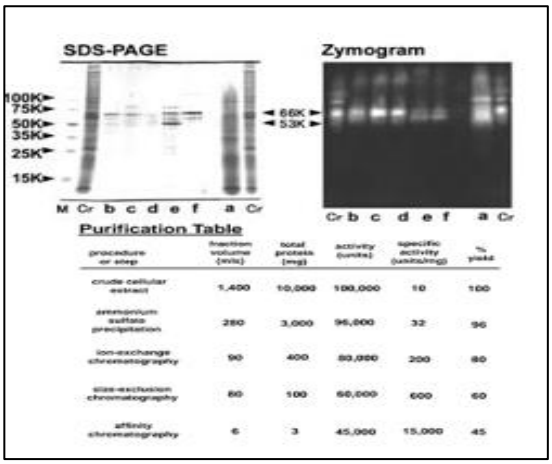
3. Purification of the α-amylase enzyme
   - □ SDS-PAGE
   - □ Fractional precipitation with metal ion
   - □ Purification table
   - □ Dialysis

3.7 Solution

Correct answer(s):

1. Size-exclusion chromatography
   - ✓ Column chromatography
   - ✓ Stationary phase contains a porous mesh of inert spherical particles
   - ✓ Mobile phase contains buffer solution
2. The amylolytic enzyme α-amylase
   - ✓ *Bacillus subtilis*
   - ✓ Randomly cleaves the 1,4-α-D-glycosidic linkages between adjacent glucose units in the amylose chain
3. Purification of the α-amylase enzyme
   - ✓ Dialysis

Session 4 : Evaluation of purification



procedure or step	fraction volume (ml/s)	total protein (mg)	activity (units)	specific activity (units/mg)	% yield
crude cellular extract	1,400	10,000	100,000	10	100
ammonium sulfate precipitation	280	3,000	96,000	32	96
ion-exchange chromatography	90	400	80,000	200	80
size-exclusion chromatography	60	100	60,000	600	60
affinity chromatography	6	3	45,000	15,000	45

- Purification table
- SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)
- Zymogram

4.1 Introduction

The evaluation of purification refers to the process of assessing the effectiveness and efficiency of purification techniques used to isolate a target protein or enzyme from a complex mixture.

After the production, extraction, isolation of extracellular amylolytic enzymes from *Bacillus subtilis*, the purification efficiency was evaluated using a purification table, SDS-PAGE, and zymography. This comprehensive approach allows for a thorough evaluation of enzyme purification. The purification table quantifies improvements in specific activity and yield, SDS-PAGE confirms the physical purity of the enzyme by showing its molecular weight and homogeneity, and zymography validates the retention of enzymatic activity, ensuring that the purification process maintained both the enzyme's purity and its functionality.

4.2 Objectives

The workshop aims to achieve the following objectives:

- Assess the degree of purification using the purification chart/table.
- Evaluate the purified enzyme by SDS-PAGE.
- Detect pure α-amylase and its activity by zymography.

4.3 Products and equipment

The following **table** lists all the necessary items for this session:

Table 4.1 : Consumable and non-consumable materials for session 4.

Products	Equipment/ instruments
➢ Enzyme sample: Crude α-amylase extracts ➢ Products: • Trichloroacetic acid (TCA) • Starch • Ice • Molecular weight protein marker (IV 10 10-180KDa) • Acrylamide • bis-acrylamide • N,N,N′,N′-tetramethylethylene-	• Vortex • Incubators • Centrifuge • Micropipettes • Water bath • Rocker shaker • Fume hood/ chemistry hood • Heat block (1.5mL) • Vertical electrophoresis • Eppendorf tubes • Electrophoresis generator • Comb with wells

diamine (TEMED) • TRIS-HCl • Ammonium persulfate (APS) • Distilled water • Lugol • Glacial acetic acid • Triton X-100 • Potassium phosphate buffer (50Mm, pH7) • Methanol/butanol/isopropanol	• Beakers • Spatulas • Precision balance • pH meter

4.4 Experimental protocols

4.4.1 Evaluation of purification via a purification chart/table

4.4.1.1 Principle

Evaluation of protein purification, such as α-amylase, using a purification chart is a systematic approach to assess the degree of purification. This method tracks the progress of the enzyme's purification, identifies the step at which the highest purity is achieved, and allows for optimization of the purification process if necessary.

A purification table is used to calculate the total protein amount, the α-amylase activity (z), the specific activity (Z_{sp}), and the purification yield (Y).

4.4.1.2 Procedure

1. Complete the table below.

2. For each purification step (from crude sample to chromatography), determine the volume, total protein content, then calculate the α-amylase activity, specific activity and purification yield using the following formulas:

$$Zsp(IU/mg) = \frac{enzyme\ activity\ \ (IU)}{proteins\ quantity\ (mg)}$$

$$f = \frac{enzymatic\ activity\ of\ each\ step\ of\ purification\ \ (IU/mg)}{enzymatic\ activity\ of\ initial\ step\ (IU/mg)}$$

$$Y = \frac{total\ enzymatic\ activity\ recovered\ during\ the\ purification\ step\ (IU)}{total\ initial\ enzyme\ activity\ (IU)} \text{x } 100\%$$

Table 4.2: α-amylase purification chart.

Purification step	Sample volume (mL)	Total content protein (mg)	Amylase activity (IU)	Specific activity (IU/mg)	Purification Yield (%)
Crude sample					
Precipitation					
Size exclusion chromatography					
Affinity chromatography					

4.4.2 *Evaluation of protein purification by SDS-PAGE*

4.4.2.1 Principle

SDS-PAGE is an analytical technique used to separate proteins based on their electrophoretic mobility. When proteins are placed in an electric field, they migrate toward their respective electrodes. The electrophoretic mobility of a protein depends on its structure, size, and charge.

Under denaturing conditions, SDS imparts a negative charge to all molecules, eliminating charge as a criterion for separation. As a result, proteins are separated primarily based on their molecular weights. Smaller molecules move faster due to less hindrance, while larger molecules move slower because of greater hindrance.

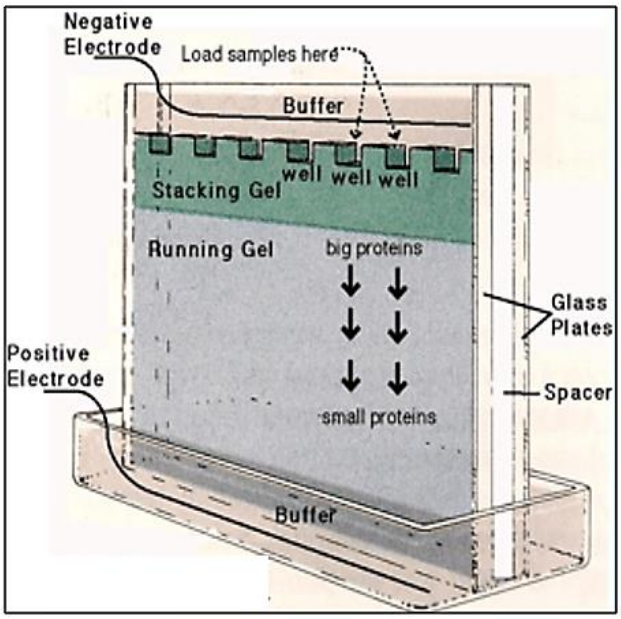


Figure 4.1: Demonstration of an apparatus used for SDS-PAGE.

4.4.2.2 Procedure

a) Preparation of SDS-PAGE gels

1. Take the mold, pour the gels, and seal the ends to prevent leaks.
2. Insert the comb to form the wells.
3. After polymerization, clamp or secure the gel in the apparatus, and fill both buffer chambers with running buffer according to the specific apparatus instructions.

Table 4.3: Composition of electrophoresis gels.

Products	**Separating gel 12% (8mL)**	**Stacking gel 5% (5mL)**
Distilled water	3.4 mL	3.5 mL
40% (Acrylamide: bis acrylamide) : (38:2)	2.4 mL	0.75 mL
Tris	2mL (1.5M, pH 8.8)	0.65 mL (1M, pH 6.8)
SDS 10%	80 µL	50 µL
APS 10%	80 µL	50 µL
TEMED (stored in refrigerator)	8 µL	5 µL

Table 4.4: Buffer compositions used for SDS-PAGE.

Pruducts	**Loading buffer 5X**	**Running buffer (1X pH 8.3)**
Tris	1.5 mL (1.5M pH 6.8)	3 g
Bromophenol blue	0.03 g	/
Glycerol	3 mL	/
β-mercaptoethanol	0.35 mL	/
SDS	0.6 g	1 g
Glycine	/	18.7 g
Distilled water	7.5 mL (V_f)	1 L (V_f)

b) Protein concentration of culture supernatant by TCA

1. Mix 100 µL of cold TCA (100%) with 1 mL of supernatant, gently vortexing as necessary to precipitate the protein.
2. Incubate the mixture for 30 min at 4°C.
3. Spin the tube in a centrifuge at 14,000 rpm for 15 min.
4. Recover and dry the pellet in the oven at 60°C.

c) Sample preparation and visualization of proteins in SDS PAGE gels

1. Add 20 µL of loading buffer to concentrate protein with TCA (pellet).
2. Incubate in a 95°C water bath for 10 min.

3. Load samples and a molecular weight protein marker into the wells to separate them by electrophoresis. Separation will first occur in the stacking gel for approximately 30 min at 100 V, followed by the separating gel for about 90 min at 120 V.

4. After separation on the polyacrylamide gel, wash the gel with 3 aliquots of water, shaking for 5 min each, and remove all water from the gel container.

5. Completely cover the gel with stain solution (add enough Coomassie stain to cover the gel) and let stain approximately for 1 h on a shaker to reveal the bands present in the gel. If the protein signal is low, stain overnight.

6. Pour off the Coomassie stain and rinse gels with water.

7. Add fresh destain solution to cover the gel for 10 min on a shaker.

8. Pour off the destain solution again, incubate for an additional 10 min to overnight, and analyze the protein bands.

Table 4.5: Staining and destaining solutions for SDS-PAGE.

Products	**Staining solution (100 mL)**	**Destaining solution (1000mL)**
Coomassie blue-R250	0.5 g	/
Methanol/Distilled water (V/V)	90 mL	900 Ml
Acetic acid	10 mL	100 mL

4.4.2.3 Reading

After separation and visualization of the bands, the relative migration distance (R_f) of the protein standards and the unknown protein can be determined. The R_f value is defined as the mobility of a protein divided by the mobility of the ion front. Since the ion front can be difficult to pinpoint, mobilities are normalized to the tracking dye that migrates just behind it:

$$Rf = \frac{distance\ to\ band}{distance\ to\ dye\ front}$$

Utilizing the R_f values obtained for the protein standards, a graph can be plotted of log MW (molecular weight) vs. R_f (**Figure 4.2**). This plot typically reveals a linear relationship for most proteins, provided they are fully denatured and the gel percentage is suitable for the molecular weight range of the samples.

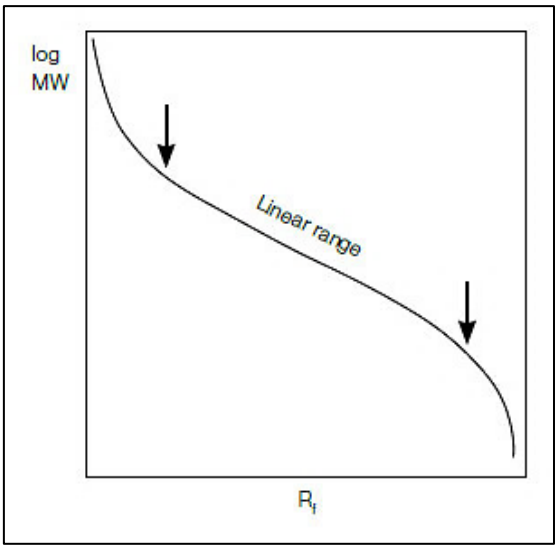


Figure 4.2: Standard protein curve of log MW vs. R_f.

4.4.3 Evaluation of purification of hydrolytic enzyme α-amylase by zymography

4.4.3.1 Principle

Zymography is an electrophoretic technique used to study hydrolases through thin gels containing copolymerized specific substrates under non-reducing conditions. This method relies on an SDS-PAGE gel impregnated with an appropriate substrate (e.g., starch for α-amylase), which is degraded by the resolved enzyme during the incubation period. This non-destructive approach permits *in situ* visualization and localization of potential enzyme activities, measured by fluorescence or colorimetry. Furthermore, enzyme activity is observed through substrate conversion and a specific stain, facilitating both identification on the gel and estimation of the enzyme's molecular weight by comparison with profiles obtained from SDS-PAGE.

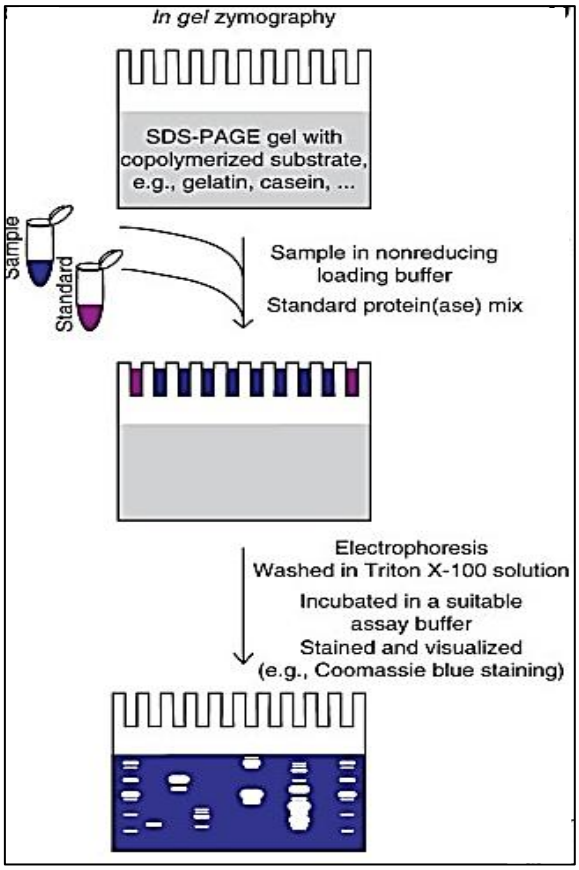


Figure 4.3: Principle of zymography.

4.4.3.2 Procedure

This approach follows similar standards as SDS-PAGE, with some modifications.

1. Prepare samples without adding reducing agents (e.g., ß-mercaptoethanol) or applying heat treatment.
2. Prepare the separation gel by adding 5 mL of starch solution (1%).
3. After the migration of the protein (enzyme) under the same experimental conditions, cover or immerse the gel with 12% TCA solution.
4. Wash the gel with 3 aliquots of Triton X-100 (2.5%), shaking for 20 min each.
5. Incubate the gel at 50°C for 16-18h, in the presence of appropriate buffer for the enzyme extract, corresponding to the pH optimum for activity.
6. Immerse the gel in Lugol's staining solution with moderate agitation, then wash with distilled water. The destaining process is the same as that used in SDS-PAGE.

4.4.3.3 Reading

The formation of clear zones against a dark background in the gel stained with iodine indicates the presence of amylolytic activity. These clear zones result from the degradation of

the substrate (starch) by α-amylase, which creates areas where the iodine does not bind, contrasting with the surrounding gel. This visualization not only facilitates the identification of enzyme activity but also helps in assessing the effectiveness of the enzyme in hydrolyzing starch. Furthermore, by comparing the positions of the clear zones to a molecular weight marker, one can estimate the molecular weight of α-amylase, providing additional insights into the enzyme's characteristics.

4.5 Conclusion

At the end of the session, each student will be able to evaluate enzymatic purification using SDS-PAGE, zymography, and purification chart to monitor and assess the purification of proteins.

4.6 Exercise

Complete the purification table based on the following data:

The enzymatic activity in the crude extract is 20000 IU, and the total protein content is 1000 mg.

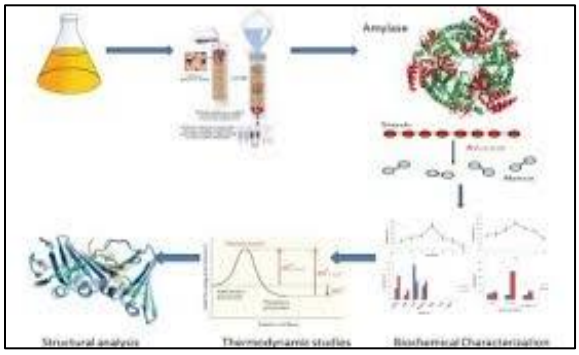
Step	Protein (mg)	Enzymatic activity (IU)	Specific activity (____)	Purifictaion level	Yield (____)
Size-exclusion chromatography	200	14000	70	_____	_____
Affinity chromatography	15	4500	_____	4.28 fold	_____

4.7 Solution

Complete the purification table :

Step	Protein (mg)	Enzymatic activity (IU)	Specific activity **(IU/mg)**	Purifictaion level	Yield **(%)**
Size-exclusion chromatography	200	14000	70	**3.5**	**70**
Affinity chromatography	15	4500	**300**	4.28 fold	**32.14**

Session 5 : Characterization of α-amylase enzyme and determination the effect of physicochemical parameters on enzyme activity and stability



- ♣ Effect of pH on α-amylase activity and stability
- ♣ Effect of temperature on α-amylase activity and stability
- ♣ Effect of incubation time on α-amylase activity and stability
- ♣ Effect of solvents on α-amylase activity and stability
- ♣ Effect of inhibitors and activators on α-amylase activity and stability
- ♣ Effect of metal ions on α-amylase activity and stability

5.1 Introduction

Characterization of purified α-amylase produced by *Bacillus subtilis* is essential for understanding its properties and optimizing its application, especially in stability studies where enzyme integrity directly impacts product yield. This process involves assessing various physicochemical characteristics that define the enzyme's functionality. As most of these biomolecules are proteins with distinct structural conformations, their activity can be influenced by factors that disrupt their structure, as well as general catalytic conditions. Many amylases are classified as metalloenzymes, demonstrating enhanced activity in the presence of divalent ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}.

5.2 Objectives

The workshop aims to achieve the following objectives:

- Determine the effect of different temperatures, and pH levels on the activity of α-amylase.
- Evaluate the effect of different solvents, inhibitors, and metal ions on amylolytic activity.

5.3 Products and equipment

The **table** below shows the materials and products most commonly used during the session.

Table 5.1 : Materials and products most commonly used during the session.

Products	Equipment/ instruments
➢ Enzyme sample: Purified α-amylase extracts ➢ Products: • Starch • Acetone • Ethylenediaminetetraacetic acid (EDTA) • Potassium phosphate buffer (50Mm) • Buffered starch (1%) • DNSA reagent • Dimethyl sulfoxide (DMSO) • Calcium chloride ($CaCl_2$)	• Vortex • pH meter • Precision balance • Incubators • Spectrophotometer • Erlenmeyer flasks • Beakers • Spatulas • Bunsen burner • Pipette tips • Micropipettes

• Cobalt nitrate II ($Co(NO_3)_2$) • Ferric trichloride or Iron (III) chloride ($FeCl_3$) • Magnesium dichloride ($MgCl_2$) • Lead nitrate ($Pb(NO_3)_2$) • Stannous chloride or Tin(II) chloride ($SnCl_2$) • β-mercaptoethanol	

5.4 Experimental protocols

5.4.1 Characterization of α-amylase enzyme

5.4.1.1 Principle

α-Amylase activity was assessed by detecting reducing sugars and proteins. To characterise the amylolytic enzymes produced by *Bacillus subtilis* strain and evaluate their optimal activity and stability, several factors were examined, including solvents, inhibitors, metal ions, pH, and environmental temperature.

5.4.1.2 Procedure

Effect of pH on α-amylase activity and stability

1. Prepare potassium phosphate buffers (50mM) with pH values ranging from 4 to 10.
2. Dissolve 1% starch in each buffered phosphate.
3. Mix 1V of purified enzyme with 1V of buffered starch (V/V).
4. Incubate the reaction mixture for 10 min at 70°C and assay for amylolytic activity. The pH stability was expressed as percent residual activity, with the initial enzyme activity at each pH considered as 100%. The relative enzyme activity at different pH were calculated using the following equation:

$$Relative\ Enzyme\ Activity\ (\%) = \frac{residual\ enzyme\ activity}{initial\ enzyme\ \ activity}\ x100$$

Effect of temperature on α-amylase activity and stability

1. Incubate the reaction mixture (1V of enzyme +1V of buffered starch solution (1%) at optimum pH) at temperature ranging from for 30 to 100°C for 10 min.

2. Assay for amylolytic enzyme activity: Samples were taken at regular time intervals of 30 min, and cooled on ice before being used to determine residual enzymatic activity under optimal conditions. The untreated enzyme is considered as control (100%).

Effect of incubation time on α-amylase activity and stability

This analysis was carried out during session 2, specifically in part 2.4.4 on amylolytic activity assay.

Effect of solvents on α-amylase activity and stability

1. Pre-incubate the enzyme-starch buffer in 10% acetone and DMSO for 10 h at optimal temperature.
2. After incubation, measure amylolytic activity.

Effect of inhibitors and activators on α-amylase activity and stability

1. As before, pre-incubate 0.09 mL of purified enzyme with 0.09 mL of buffered starch (1%) and 0.02 mL of EDTA solution (10 mM) and β-mercaptoethanol (10 mM) for 1 h at the optimal temperature.

Effect of metal ions on α-amylase activity and stability

1. Conduct the amylase assays in the presence of various metal ions ($CaCl_2$, $Co(NO_3)_2$, $FeCl_3$, $MgCl_2$, $Pb(NO_3)_2$, and $SnCl_2$) at a final concentration of 2 mM in phosphate buffer (50 mM).
2. Add 1% of starch and determine the relative enzyme activity under standard assay conditions.

5.5 Conclusion

At the end of the session, each student will be able to elucidate the optimal conditions for α-amylase activity, by determining the influence of various chemical and physical agents and factors on their activity and stability.

5.6 Exercise

Enzyme reactions are highly dependent on pH and temperature. The study of these physicochemical parameters and their effect on enzymatic activity is presented in the graphs below.

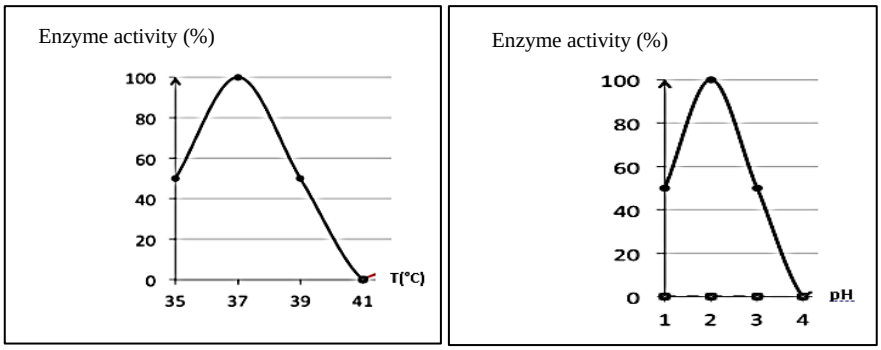


Figure 1: Effect of pH and temperature on enzyme activity.

4. What conclusions can be drawn from this study?

5.7 Solution

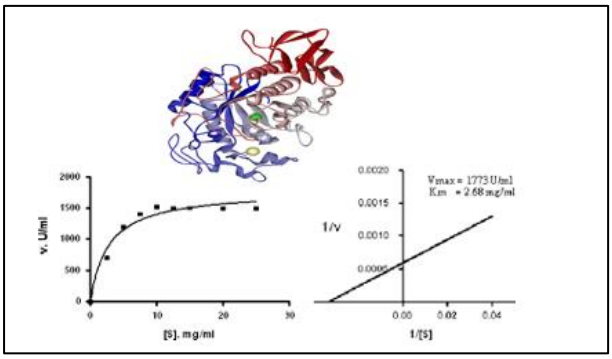
1. Conclusion:

***** Some physicochemical parameters of the enzyme include:

• An optimal temperature of 37°C.

• An optimal pH of 2.

Session 6 : Determination of enzyme kinetics



- ♣ Enzyme kinetic parameters
- ♣ Debate and closure

6.1 Introduction

Enzyme kinetics is the study of chemical reactions hydrolyzed by enzymes. In this field, the reaction rate is measured, and the effects of varying conditions on the reaction are investigated. The Study of these parameters provides essential information about the rate, efficiency, and specificity of enzymatic reactions, leading to a deeper understanding of enzyme functionality.

6.2 Objectives

The workshop aims to achieve the following objectives:

- Evaluate the kinetic parameters using the **Lineweaver-Burk** representation.

6.3 Products and equipment

The main equipment and products used in the session are listed in **table 6.1.**

Table 6.1 : Main equipment and products used during the session.

Products	Equipment/ instruments
➢ Enzyme sample: Purified α-amylase extracts ➢ Products: • Starch • Potassium phosphate buffer (50Mm) • DNSA reagent	• Spectrophotometer • Tubes • Pipette tips • Micropipettes • Water bath

6.4 Experimental protocols

6.4.1 Determination of enzyme kinetic parameters

6.4.1.1 Principle

The kinetic parameters of purified α-amylase are determined graphically using the **Lineweaver-Burk** plot. Soluble starch at several concentrations (0.2 to 1 mg/mL) serves as the natural substrate for the purified enzyme under optimal conditions.

The main kinetic parameters of enzyme activity include the initial rate (V_i), which is the rate measured or calculated at the beginning of the reaction; the Michaelis constant (K_M), representing the substrate concentration at which the reaction rate reaches half of its maximum value; and the maximum rate or maximum initial reaction velocity (V_{max}), which indicates the enzyme's maximum capacity to catalyse the reaction.

These kinetic parameters are essential for elucidating enzymatic mechanisms and are typically determined experimentally using methods such as spectrophotometry.

6.4.1.2 Procedure & Reading

1. Prepare starch concentrations ranging from 0 to 1 mg/mL in phosphate buffer (starch buffered, 50 mM).
2. Preheat the buffered starch solution to 70°C.
3. Mix α-amylase enzyme with buffered starch.
4. Incubate tubes at 70°C at 10 min.
5. Stop the reaction by adding 2V of DNSA to each tube.
6. Boil the mixture at 100°C for 5 min.
7. Measure the concentration of reduced suagar by DNSA method at 540 nm.
8. Determine the kinetic parameters (K_M and V_{max}) of α-amylase enzyme from the resulting curve.

6.5 Conclusion

At the end of the session, each student will be able to determine key enzyme kinetic parameters and interpret the data to understand the dynamics of enzyme-catalyzed reactions.

6.6 Exercise

Fill in the blanks:

1. The Lineweaver-Burk plot is a graphical representation of the of enzyme kinetics.
2. The kinetic parameters can be determined by incubating the enzyme with ,using a suitable equation.
3. KM is a measure of the concentration at which an enzyme attains of its maximum velocity.

6.7 Solution

Fill in the blanks:

4. The Lineweaver-Burk plot is a graphical representation of **Michaelis–Menten** the of enzyme kinetics.

5. The kinetic parameters can be determined by incubating the enzyme with **different substrate concentrations,** using a suitable equation.

6. K_M is a measure of the **substrate** concentration at which an enzyme attains **half** of its maximum velocity.

Debate and closing day

During the session, instructors will begin by outlining the expectations and assessment criteria. Afterwards, a discussion will take place to encourage students to present their results and calculated data, thus promoting the exchange of ideas. The dialogue will also explore the implications of their findings and the challenges encountered during the workshop, providing an opportunity for reflection and a deeper understanding of enzymes and their applications in industrial fields.

Conclusion

The enzyme engineering workshop integrated various scientific disciplines, including microbiology, biochemistry, and enzymology. It focused on the bacterial enzyme α-amylase (approximately 45 kDa), produced by *Bacillus subtilis* by submerged fermentation and recovered by centrifugation. The purified enzyme was evaluated by electrophoresis and further characterized to optimize its activity and stability.

The scientific and technical knowledge acquired throughout this workshop provides students with practical skills essential for professional practice. By simulating real-world industrial environments, the workshop helps them better understand the industry demands or requirements and prepares them to apply their expertise in future careers.

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