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Second cycle Department

Educational handout

Subject: Workshop

Purification and characterization of enzymes

Level: 2nd year of the second cycle
Field: Biological Sciences
Speciality: Enzyme engineering
Domain: Natural and Life Sciences

Prepared by:

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Subject taught during the Academic Years:

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List of abbreviations

- **APS:** ammonium persulfate
- **DNSA:** 3,5-dinitrosalicylic acid
- **ANSA:** 3-amino-5-nitrosalicylic acid
- **D W:** distilled water
- **Km:** Michaelis constant
- **SDS-PAGE:** sodium dodecyl sulfate polyacrylamide gel electrophoresis
- **Smf:** submerged fermentation
- **TCA:** trichloroacetic acid
- **TEMED:** N,N,N′,N′-tetramethylethylenediamine
- **n:** number of moles
- **EDTA :** ethylenediaminetetraacetic acid
- **$CaCl_2$**: calcium chloride
- **$Co(NO_3)_2$**: cobalt(II) nitrate
- **$FeCl_3$**: iron(III) chloride
- **$MgCl_2$**: magnesium chloride
- **$Pb(NO_3)_2$**: lead(II) nitrate
- **$SnCl_2$**: tin(II) chloride

Foreword

This report was developed within the framework of the **Enzyme Purification and Characterization** module, part of the **Enzyme Engineering** curriculum. It provides a structured scientific and technical overview of the practical laboratory procedures conducted, closely aligned with the theoretical principles covered during the course.

The main objective of this work is to apply commonly used methods in the field of biochemistry and enzyme engineering, particularly those related to enzyme purification, assessment of catalytic activity, and characterization of physicochemical properties. Through techniques such as precipitation, dialysis, and chromatography, the aim is to monitor the purity of the enzyme throughout the purification process while measuring its activity at different stages.

This experimental approach not only reinforces theoretical knowledge but also helps develop practical skills essential in the biotechnology field. Methodological rigor, adherence to protocols, critical analysis of results, and mastery of analytical tools are all key elements emphasized during this practical experience.

Particular attention is given to the clear presentation of results, their scientific interpretation, and the ability to relate experimental findings to core theoretical principles. This document reflects the importance of a balanced education between theory and practice and highlights the growing relevance of enzymes as key tools in various industrial, pharmaceutical, and environmental applications.

Workshop information

Institution: Higher School of Biological Sciences of Oran

Department: Second Cycle

Option: Enzyme Engineering

Target Audience: 2nd Year Enzyme Engineering Students

Unit: UEM 1

Credits: 05

Coefficient: 03

Duration: 12 weeks

Time spent: 1 semester

Schedule: Thursday (08h30-15h30)

Location: Enzyme Engineering Laboratory

Practical Leader: Dr. KHODJA. Badra

Practical Instructor: Dr. AIT HAMADOUCHE Yasmin

Contact: by e-mail at khodjabadra@hotmail.com

Availability: In the teacher's room (Monday from 11:00 to 12:00).

Workshop presentation

Enzyme engineering represents an eminently strategic field within contemporary biotechnology, aimed at improving the performance or conferring new functionalities on specific enzymes or proteins. Enzymes, natural protein molecules, play a crucial role as biological catalysts, orchestrating and accelerating chemical reactions within living organisms. To fully exploit the potential of these biomolecules, protein purification becomes an essential step. This complex process involves a series of techniques designed to isolate and purify a specific enzyme or protein complex from diverse sources, including cells, tissues, or even whole organisms.

The purification of proteins is proving to be a fundamental prerequisite for their in-depth characterization and use in various fields of application, including basic and applied research as well as medicine. With this in mind, this workshop proposes to follow in detail the path of the enzyme α-amylase, from its initial production to its purification, using a range of varied methods such as precipitation and advanced chromatographic techniques, notably exclusion chromatography. Careful evaluation of the purification process is essential to assess the progress made in isolating this essential enzyme.

Subsequently, a detailed analysis of physicochemical parameters will provide a better understanding of the intrinsic characteristics of the α-amylase enzyme, while the study of its kinetics will provide crucial insights into its catalytic properties and efficiency under specific conditions. This methodical, in-depth work on the α-amylase enzyme will thus further our understanding of enzymatic mechanisms, while opening the way to potential new applications in fields as varied as biotechnology, the food industry, and pharmacology.

Contents

This workshop program comprises five workshops designed to provide students with the fundamentals necessary to master the enzyme purification process and the various parameters used to evaluate purification efficiency.

Protein purification is a series of processes designed to isolate a protein from a complex mixture. The purification of enzymes is necessary for their characterization and for their application in fields such as research and medicine.

In this context, the workshop is scheduled as follows:

1- Opening and explanation. Reminder of laboratory safety.

2- Preparation of solutions and culture media.

3- Production of the enzyme α-amylase by fermentation.

4- Fractional precipitation of α-amylase enzyme with ammonium sulfate.

5- Removal of ammonium sulfate by dialysis.

6- Exclusion chromatography.

7- Affinity chromatography.

8- Purification evaluation (purification table, SDS-PAGE, zymogram).

9- Characterization of the alpha-amylase enzyme and determination of the effect of physico-chemical parameters on enzyme activity and stability.

10- Determination of the enzyme's kinetic parameters.

11- Evaluation, discussion and closing.

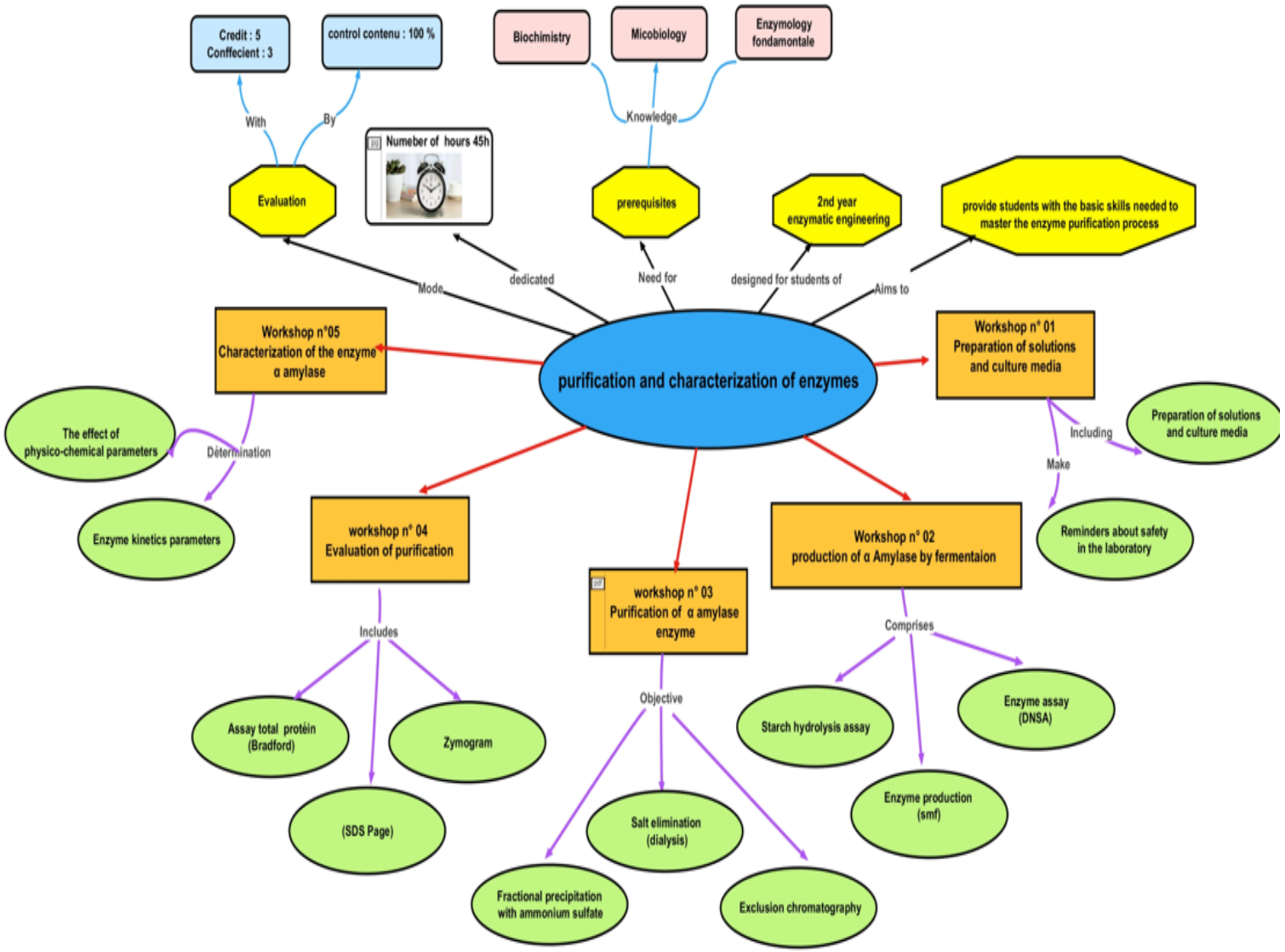


Figure 1: Concept map of workshop (Khodja B, 2024).

Prerequisites

Biochemistry: Structure and function of biomolecules, enzymes, and enzymatic kinetics,

Microbiology: Structure and function of microbial cells, microbial growth,

Biotechnology: Basic biotechnology techniques,

Biochemical analysis techniques: Protein and enzyme determination.

General introduction

Enzyme engineering is an eminently strategic field within contemporary biotechnology, aimed at improving enzyme performance or conferring new functionalities on specific enzymes or proteins (Su *et al*., 2024; Schallmey *et al*., 2023). Enzymes, naturally occurring protein molecules, play a crucial role as biological catalysts, orchestrating and accelerating chemical reactions in living organisms with high specificity and efficiency (Nelson & Cox, 2021). To fully exploit the potential of these biomolecules, protein purification becomes an essential step. This complex process encompasses a series of techniques aimed at isolating and purifying a specific enzyme or protein complex from diverse sources such as cells, tissues, or whole organisms (Scopes & Smith, 2022). The purification of proteins is a fundamental prerequisite for their in-depth characterization and application in various fields, including basic and applied research as well as medicine (Harrison *et al*., 2020). With this in mind, this workshop proposes to follow in detail the pathway of the enzyme α-amylase, from its initial production to its purification, using a range of methods such as precipitation and advanced chromatographic techniques, notably size-exclusion chromatography (Urh *et al*., 2023). Careful evaluation of the purification process is essential to assess the progress achieved in isolating this enzyme and to determine its purity and yield. Subsequently, a detailed analysis of physicochemical parameters such as optimal pH, temperature, and molecular weight will provide a better understanding of the intrinsic characteristics of α-amylase. In parallel, the study of its enzymatic kinetics will offer crucial insights into its catalytic properties and efficiency under specific conditions (Cornish-Bowden, 2022). This methodical and in-depth investigation of α-amylase contributes to a deeper understanding of enzymatic mechanisms and opens the way to potential applications in biotechnology, the food industry, and pharmacology. The global enzyme market is dominated by hydrolases, which represent nearly 80% of commercially used enzymes, particularly amylases and proteases (Markets, 2023). Among them, α-amylases are among the most important enzymes on an industrial scale, making them key tools in modern biotechnology due to their extensive use in starch processing, food production, and fermentation industries (Gupta *et al*., 2022; Kumar *et al*., 2024).

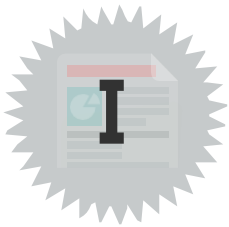
Objectives of the Workshop

This workshop aims to introduce students to the fundamental techniques of enzyme purification and characterization, encouraging them to work independently, rigorously, and safely.

By the end of this workshop, the student will be able to:

- Apply laboratory safety rules and perform experiments under sterile and controlled conditions.
- Implement basic biochemistry techniques for the production, purification, and characterization of an enzyme (α-amylase).
- Analyze experimental results at each stage using both quantitative and qualitative tools.
- Evaluate the effectiveness of an enzyme purification protocol through scientific indicators (yield, specific activity, SDS-PAGE, zymogram).
- Develop critical scientific thinking by proposing improvements and integrating results into a coherent synthesis.

Opening and Explanation: Laboratory Safety Reminders



1. Introduction

Enzymes facilitate a wide range of biochemical reactions, both in biological systems and industrial processes, and their study, production, and application require precise experimental conditions based on rigorous laboratory techniques (Kumar *et al.*, 2024). Among these techniques, the preparation of solutions and culture media represents a fundamental step in ensuring reproducible and meaningful results. Solutions provide a suitable chemical environment that maintains enzymatic stability and supports the proper course of reactions by controlling factors such as pH and ionic strength. Culture media play a crucial role in the growth, isolation, and production of microorganisms capable of synthesizing enzymes of interest by providing essential nutrients, buffering capacity, and selective conditions tailored to specific microbial needs. These media are composed of nutrients, buffers, stabilizing agents, and sometimes selective inhibitors or indicators, effectively recreating the optimal physicochemical conditions for microbial development, such as pH, isotonicity, or redox potential. Preparation of culture media can be carried out using commercially available dehydrated formulations, individual components, or ready-to-use media, depending on experimental needs and the microbial species involved. This process typically includes several key steps: dissolving ingredients in distilled or deionized water, adjusting pH to the desired level, distributing the solution into appropriate containers, sterilizing (often by autoclaving), and storing under controlled conditions to preserve sterility and effectiveness (Sigma-Aldrich culture media preparation, 2025; FAO protocol on media preparation, 2025). Rigorous preparation of media and solutions is therefore essential to ensure the reliability and reproducibility of microbiological experiments, particularly in fields such as research, diagnostics, quality control, and industrial enzyme production. Proper media preparation minimizes contamination risks and ensures that microbial cultures behave predictably under defined conditions (Sigma-Aldrich microbial media guidelines, 2025). Mastering these techniques provides the foundation necessary for effective enzyme characterization and successful purification of target biomolecules.

2. Specific objectives

- Presentation of the program and the activities planned during the workshop
- Apply good sterility and safety practices in the laboratory.
- Understand the role of culture media and solutions in experiments.

3. Reminder of laboratory safety

- To prevent/avoid accidents, students are required to adhere to these guidelines strictly:
- Wear a closed, long-sleeved lab coat.
- Wear goggles and a mask when there is a risk of splashing biological fluids, reagents, or culture media.
- Long hair must be tied back during handling to avoid accidental burns or contamination.
- Eating, drinking, and smoking are strictly prohibited in the laboratory.
- Light the Bunsen burner to create the sterile zone.
- Clean the lab bench with diluted bleach.
- Wash your hands thoroughly before and after handling any work and before leaving the laboratory, even briefly.
- Do not touch the mouth or face when handling.
- Put all the equipment back in its proper place and arrange the chairs.

Table 1: Safety pictograms and risk indications

Products	**Hazard pictograms**	**Warnings**
Sodium hydroxide		Corrosive
Hydrochloric acid		Corrosive harmful and irritating
Acetic acid		Skin irritation and serious eye damage
Ethanol		Highly inflammable

Preparation of solutions and culture media

1. Introduction

The preparation and use of culture media, buffers, and reagents require careful attention to their composition and handling to ensure reliable and reproducible results. Solid media containing agar must be heated to completely dissolve the agar before sterilization. All media should be sterilized in an autoclave at 121 °C for 20 minutes to eliminate any contamination. The pH of the media must be properly adjusted before sterilization. Buffers should be carefully prepared to stabilize biochemical reactions, and reagents must be properly stored to preserve their integrity.

2. Specific objectives

- Accurately carry out the essential steps in preparing culture media and solutions.

3. Experimental protocols

3.1. Materials

Products	Equipments
• Ortho phosphoric acid • Potassium sodium tartrate tetrahydrate • DNSA • NaOH • Potassium phosphate buffer • HCl • Tris-SDS • Ammonium sulfate • Coomassie Blue G250 • Ethanol • Potassium phosphate buffer	• pH meter • Precision balance • Refrigerator • Bunsen burner • Vortex • Micropipettes • Autoclave • Incubators

Table 2: Composition of media, solutions and buffers

Reagents and buffers		**Culture media**	
Compound	**Amount**	**Compound**	**Amount**
DNSA Reagent (3,5-dinitrosalicylic acid) (200mL) • DNSA • NaOH (2M) • Potassium sodium tartrate tetrahydrate • Distilled water	 10g 16g 300g 1L	**Dehydrated broth media: LB+ 1% starch, soluble, potato** (300 mL) • Peptone • Yeast extract • Sodium chloride • Distilled water pH7	 10g 5g 5g 1L
Buffer potassium phosphate, 50 mM, pH 7,0 (4L) • Monopotassium phosphate (KH_2PO_4) • Dipotassium phosphate (K_2HPO_4) • Distilled water	4,672g 3,154g 1L	**Dehydrated agar media: LB + 1% starch, soluble, potato** (150 mL) • Peptone • Yeast extract • Sodium chloride • Agar agar • Distilled water pH 7	 10g 5g 5g 15g 1L
Dialysis tubing preparation • Sodium bicarbonate solution • EDTA	2% (w/v) 1mM	**Dehydrated broth media: LB** (50 mL) • Peptone • Yeast extract • Sodium chloride • Distilled water pH 7	 10g 5g 5g 1L
Bradford reagent (200mL) • Coomassie Blue G-250 • Ethanol 95% • Orthophosphoric acid 85% • Distilled water	5mg 2.5mL 5mL 50 mL	**Dehydrated agar media: LB (**50mL) • Peptone • Yeast extract • Sodium chloride • Agar agar • Distilled water pH 7	 10g 5g 5g 15g 1L

Table 3: Buffer compositions for SDS-PAGE

Compound	Amount	Compound	Amount
TRIS buffer(1MpH6.8) • TRIS • Distilled water	6,057g 50ml	**Running buffer (1X ph8.3)** • TRIS • SDS • Glycine • Distilled water	3g 1g 18,7g 1L
TRIS buffer(1.5MpH8.8) • TRIS • Distilled water	9,435g 50ml	**Loading buffer5X** • **Tris** (1 M pH=6.8) • Bromophenol blue • Glycerol • β-mercaptoethanol • SDS • Distilled water (Vf)	1,5ml 0,03g 3ml 0,35ml 0,6g 7,5ml
Staining solution (100mL) • Coomassie blue (R250) • Methanol/distilled water(v/v) • Glacial acetic acid	0,5g 90ml 10ml	**Destaining solution**(1000mL**)** • Glacial acetic acid • Methanol/distilled water (v/v)	100ml 900ml
• SDS 10 % • Ammonium persulphate (APS) 10 %	10 ml 10ml	• HCl 4N • NaOH2M	100ml 100ml

Production of α-amylase enzyme by fermentation

III

1. Introduction

The α-amylases are hydrolytic enzymes that are widely distributed in nature, being present in animals, microorganisms, and plants. They play a fundamental role in the hydrolysis of starch and other polysaccharides, which makes them enzymes of great biological and industrial importance. Due to their functions, they are among the most studied and exploited enzymes in biotechnology. Indeed, with the advances achieved in this field, a wide range of microbial α-amylases has been identified and applied in several industrial sectors. Their applications include the food industry (bread, beer, and glucose syrup production), fermentation (bioethanol and other bioproducts), textile (fabric treatment), paper (improvement of pulp and paper quality), detergents (formulation of enzymatic laundry products), as well as the pharmaceutical industry (formulation of drugs and enzyme supplements) **(**Pandey *et al*., 2000; Gupta *et al*., 2003; Kandra, 2003).

Figure 2: α-amylase applications **(**Mehta D, 2016).

In this context, the present study focuses particularly on the amylase produced by the bacterial strain *Bacillus subtilis*. This Gram-positive, catalase-positive bacterium is commonly found in soil, where it plays an important ecological role (Earl *et al*., 2020). As a member of the genus *Bacillus*, *B. subtilis* is distinguished by its ability to form resistant and protective endospores, an adaptive mechanism that enables it to survive under extreme environmental conditions such as drought, heat, or exposure to certain chemical agents (Tan & Ramamurthi, 2021). Beyond its ecological importance, *B. subtilis* holds a prominent place in the industrial field due to its remarkable capacity to produce a wide variety of proteins, including high-value extracellular enzymes (Schallmey *et al*., 2023). Its efficient secretion system, safety of use (generally regarded as non-pathogenic), and biotechnological potential make this bacterial species a host of choice for industrial enzyme production (van Dijl & Hecker, 2019; Su *et al*., 2024). Among these enzymes, the α-amylase from *B. subtilis* stands out as a particularly promising candidate, offering considerable potential for diverse applications in modern bioprocesses (Gupta *et al*., 2022; Kumar *et al*., 2024).

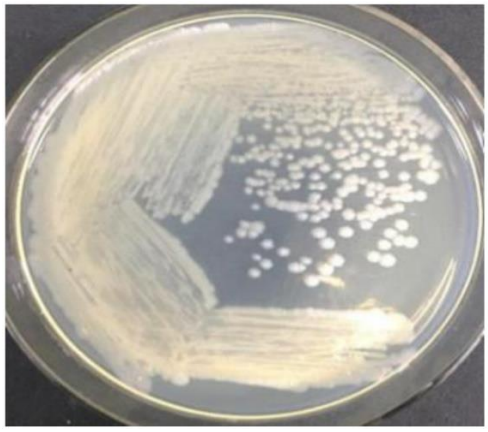


Figure 3*: Bacillus subtilis* on growth medium (Ju *et al*.,2019).

2. Specific objectives

The workshop aimed to achieve the following objectives:

- Determine the ability of a microorganism to hydrolyze starch,
- Production of α-amylase enzyme by submerged fermentation (Smf),
- Extract the enzyme by centrifugation,
- Determine amylolytic activity by the Miller method,
- Quantify proteins by the Bradford method.

3. Experimental protocols

3.1 Materials

Products	Equipments
• Distilled water • Ice cube • Lugol • Iodine crystals • Starch, soluble, potato • DNSA reagent • Bradford reagent • Maltose • LB media • BSA	• Micropipettes • Erlenmeyer flasks • Centrifuge • Bunsen burner • Petri plates • Yellow pipette tips • Test tubes • Incubators • Water bath • Spectrophotometer

3.2 Starch hydrolysis test (amylase test)

Principle *Review*

The starch hydrolysis test is based on the ability of certain microorganisms to produce extracellular enzymes (mainly α-amylases) capable of degrading starch, a polysaccharide composed of amylose and amylopectin**.**

Amylose is a linear polysaccharide made up of glucose units linked by α-(1→4) glycosidic bonds, while **amylopectin** is a highly branched polysaccharide consisting of α-(1→4) linkages in the main chains and α-(1→6) linkages at the branching points.

Starch incorporated into a solid culture medium (usually nutrient agar containing 1% soluble starch) serves as the substrate. During incubation, if the bacterial strain produces amylases, these enzymes diffuse into the medium and hydrolyze the surrounding starch into simpler sugars (maltose, maltotriose, glucose). **(**Jahir *et al.*, 2011).

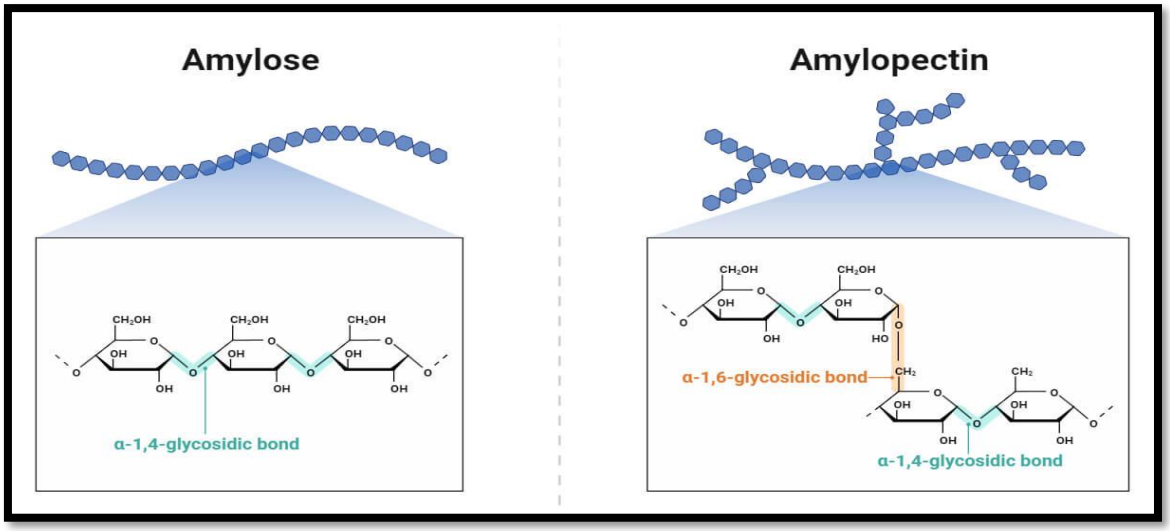


Figure 4: Starch structure **(**Jahir *et al.*, 2011).

Method

1. Make a twice streak inoculation of the microorganism to be tested in an LB starch plate (Figure 5).
2. Incubate bacteria culture for 24 hours at 37°C.
3. Following incubation, add the iodine crystals or Lugol's solution to the surface of the plates, using one of the following methods:

1st method: Flood the surface of the agar plates with **Lugol's solution** for **30 seconds.**

2nd method: Expose the bacterial colonies grown on starch agar plates to iodine vapors generated by the sublimation of crystals placed in a beaker for **30 minutes**. (Wisdomkofi *et al.*, 2006).

4. Examine for the clear zone around the line of bacterial growth.

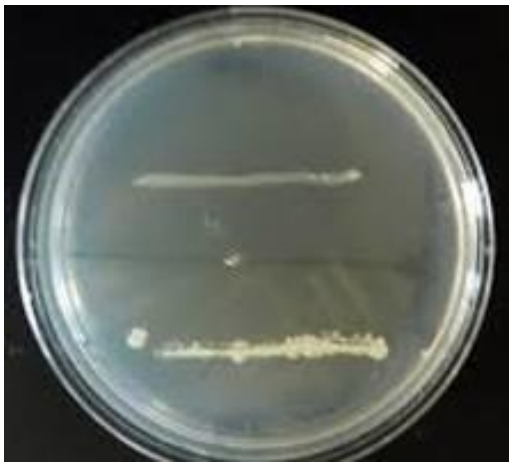


Figure 5*:* Hydrolysis Test Technique (Khodja B, 2024).

- **Positive test (Amylase +):** appearance of a **clear halo** around the bacterial growth, indicating starch hydrolysis (no color development) (Figure6).
- **Negative test (Amylase –):** the medium develops a **blue, purple, or black** coloration after the addition of iodine, indicating the presence of non-hydrolyzed starch.

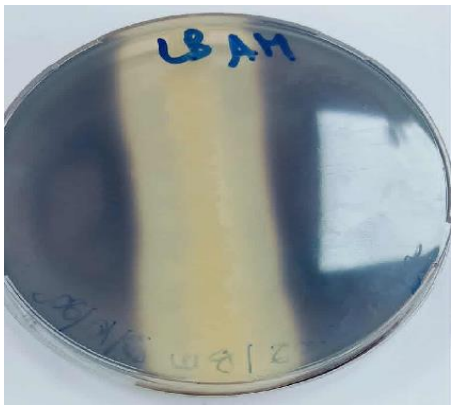


Figure 6: Hydrolysis test reading (Khodja B, 2024).

3.3 Production of α-amylase by submerged fermentation (Smf)

Principle Review

Submerged fermentation involves the growth of the microorganism as a suspension in a liquid medium (the **biomass** is immersed in a liquid medium) in which various nutrients are either dissolved or suspended as particulate solids in many culture media to allow good homogenization of the medium's components.

Method

1. Carry out an 18h pre-culture for Bacillus subtilis on 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. These broths are contained in 100mL Erlenmeyer flasks.
3. Incubate cultures overnight in a shaking incubator set to 37°C and 150 rpm.(Lin *et al*., 2025).

Figure7: Impaired bacterial growth (Khodja B, 2024).

Reading — *Complément*

The **production** of the Amylase production is indicated by a **change in the color** of the broth (**opaque**) and the appearance of cloudiness

- **Amylase production is indicated by:** a change in the color of the broth and the appearance of turbidity (cloudiness).
- **No amylase production:** the broth remains unchanged and clear.

3.3.1 Extraction of α-amylase enzyme (centrifugation and recovery of active enzymes)

Principle — *Review*

The extraction of α-amylase is based on the centrifugation of the bacterial culture medium, which removes the cells and allows the recovery of the supernatant (Gupta *et al*., 2022). This supernatant, known as the crude enzymatic extract, serves as the source of active α-amylase and is commonly used as the starting material for subsequent purification steps in enzymology and industrial bioprocesses (Li *et al*., 2023; Kumar *et al*., 2024).

Method

After incubation, the culture was centrifuged at 5000rpm for 20min at 4°C, and the supernatant was recovered and stored at -20°C.

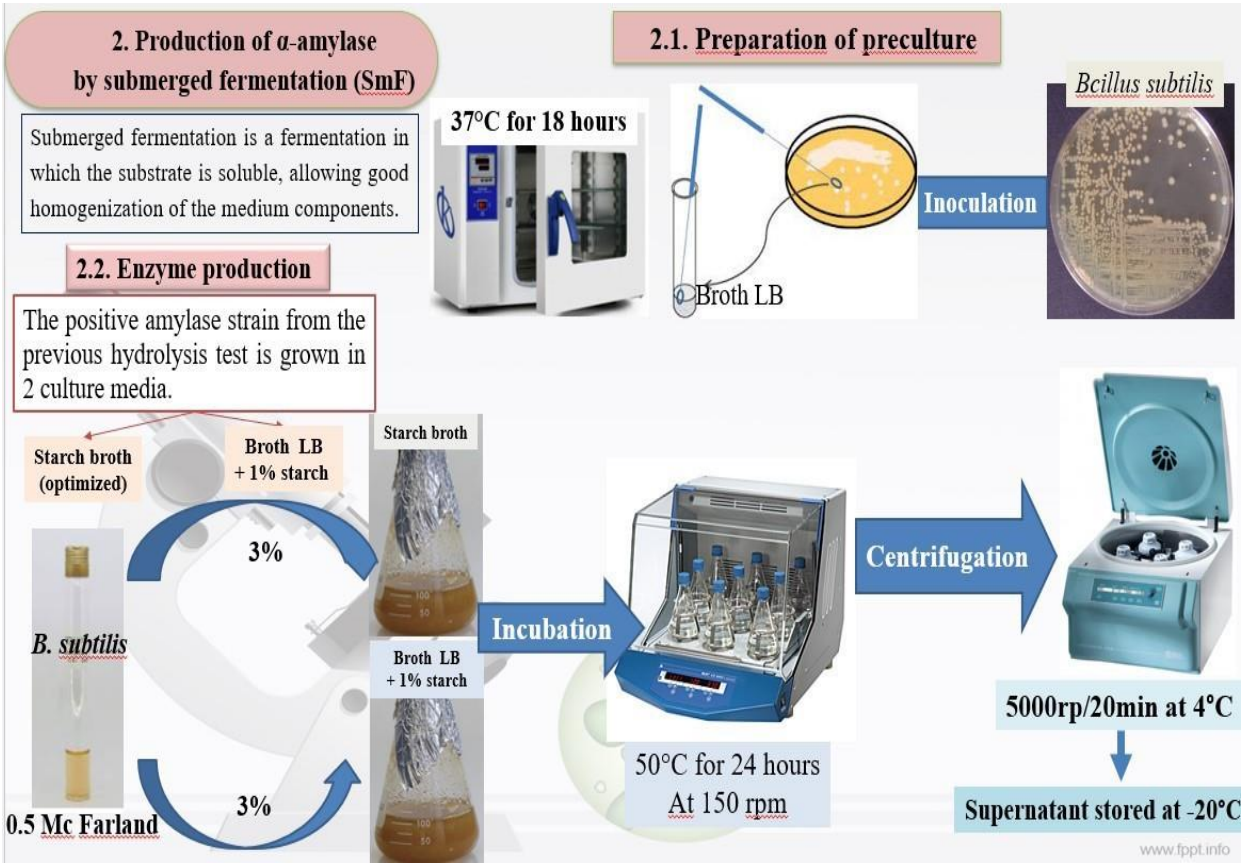


Figure 8: Production and extraction of α-amylase enzyme (Pandey *et al.*, 2000).

The next steps consist in evaluating the enzyme extract by using sugar and protein assay methods

3.4 Amylolytic activity assay

Principle *Review*

The α-amylase activity is measured using a colorimetric method of Miller (1959**)** with *DNSA* reagent (Figure 9).

In this method, starch by α-amylase is converted into maltose. This is measured by the reduction of DNSA. Therefore, the presence of a free carbonyl group (C=O) of reducing sugars is detected.

This involves the oxidation of the aldehyde functional group (−CH=O) and the ketone functional group (R−C(=O)−R'). During this reaction, *DNSA* is reduced to *ANSA*.

Under alkaline and heat conditions, the *DNSA* is converted to a reddish or orange coloured complex which has an absorbance maximum of 540 nm.

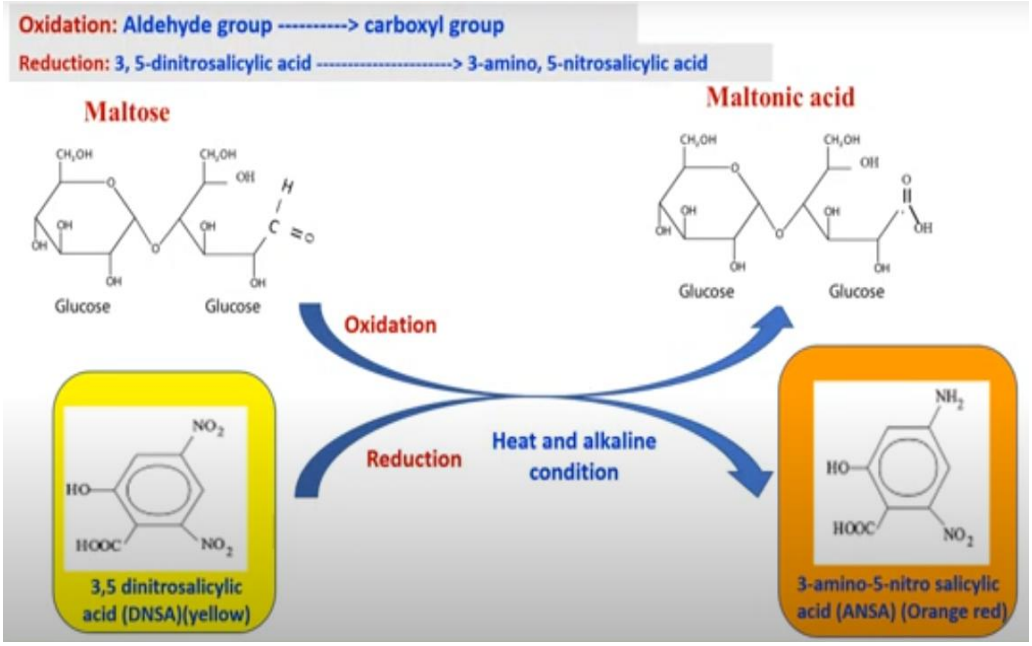


Figure 9: Reducing sugar by the DNS method (Miller,1959**).**

a) Calibration/Standard range

method

1. Prepare standards containing maltose concentrations from 0,2 to 2 mg/mL, with a final volume of 1 mL.
2. Mix 1V of each standard with 1V of DNSA reagent.
3. Keep the standards at a boiling water bath for 5 min.
4. Read absorbance at 540nm.
5. Draw a standard curve by submitting serial dilutions of maltose.

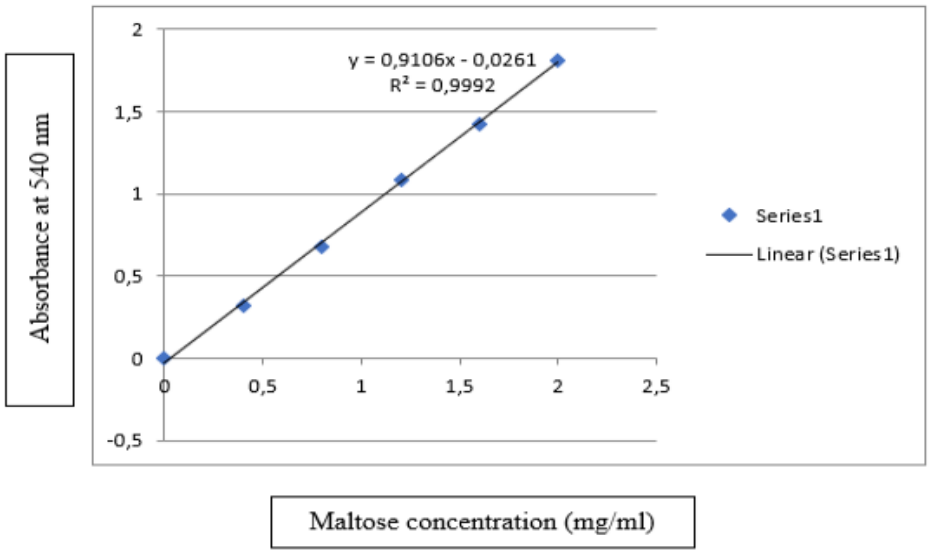


Figure 10: Maltose standard calibration curve (Khodja B, 2024).

b) Enzymatic assay of α-amylase: effect of incubation time on α-amylase production) *method*

1 Prepare 1% buffered starch

2 Mix 1V supernatant with 1V buffered starch (V/V).

3 Incubate tubes at 37°C, then take the samples every 5 min (t5 to t30). For tube 0 (t0), the sample is not incubated.

4 add 2V DNSA to each tube.

5 Incubate samples in a water bath at 100°C for 5 min or 98°C for 10 min.

6 Measure the amount of reduced DNSA at 540 nm.

7 Determine the maltose concentration (mg/mL) through the calibration curve.

8 Calculate the enzyme activity in international units (IU).

3.5 Proteins assay

Principle *Review*

The **Bradford protein assay** is one of several protein **quantification methods**. The protein content in crude enzyme preparations was determined by the method of *Bradford* (1976).

The principle of the colorimetric method is based on the reaction between the side-chain chemical groups of various amino acids with Coomassie brilliant blue reagent (G250) to give a purple-blue color with a maximum absorbance located at a wavelength of 595 nm.

a) Calibration/Standard range *method*

1 Prepare 1mg/mL *BSA*.

2 Establish a standard range containing between 0 µg and120 µg *BSA*.

b) Enzyme assay *method*

1 Mix 20 µL of the sample to be tested with 980µl of potassium phosphate buffer (50mM, pH=7).

2 Add 1 mL Bradford reagent (sample and standards).

3 Incubate the mixture at room temperature in a dark place for 15-30 min.

4 Read absorbance at 595 nm.

5 Create a standard curve by graphing the following data (Absorbance vs. Protein quantity (µg).

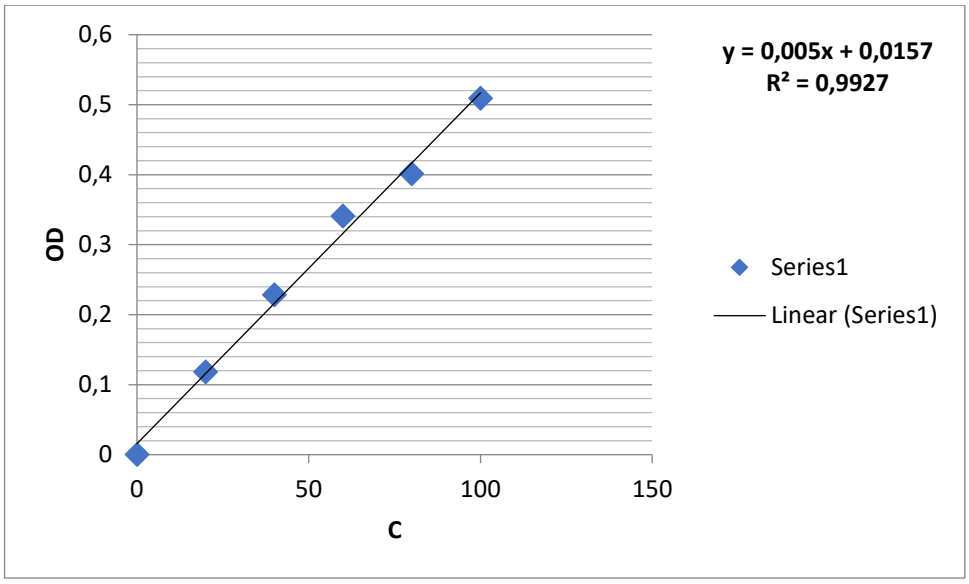


Figure 11: BSA standard calibration curve (Khodja B, 2024).

Fractional precipitation of the enzyme α-amylase with ammonium sulfate

1. Introduction

Enzyme purification is an essential step in biochemical research and industrial production. It aims to separate the enzyme of interest from other components of the solution, such as unwanted proteins, salts, metabolites, etc., and thus achieve a concentrated and active preparation for further study or use (Ezenwelu *et al.*, 2022). There are several methods of enzyme purification, each based on specific properties of the enzyme to be purified (Rahim *et al.*, 2025). Fractional ammonium sulfate precipitation is used to precipitate proteins. It is based on the principle of salting-out: in highly saline solutions, the solubility of most proteins is reduced, causing them to aggregate and precipitate (Journal of Bioscience and Applied Research, 2024). Precipitation with ammonium sulfate leads to the presence of salt around the protein, which can interfere with certain downstream techniques such as electrophoresis or activity assays, making it necessary to remove all traces of these compounds from enzyme preparations (Frontiers *et al.*, 2025).

Salt removal or desalination can be carried out either by dialysis, a technique for purifying and concentrating macromolecules (e.g., enzymes) through a semi-permeable membrane that allows salts to diffuse out (Journal of Bioscience and Applied Research, 2024), or by gel filtration (size-exclusion) chromatography using media such as Sephadex G-25, which enables rapid and simple separation of molecules based on their size and molar mass (Frontiers *et al.*, 2025).

2. Specific objectives

- Apply fractional precipitation of the enzyme α-amylase with ammonium sulfate.

3. Experimental protocols

3.1 Materials

Products	Equipments
• Distilled water • Ice cube • Lugol • Iodine crystals • Starch, soluble, potato • DNSA reagent • Bradford reagent • Maltose • LB media • BSA	• Micropipettes • Erlenmeyer flasks • Centrifuge • Bunsen burner • Petri plates • Yellow pipette tips • Test tubes • Incubators • Water bath • Spectrophotometer

3.2 Precipitation of α-amylase by ammonium sulfate (salting-out technique)

Principle *Review*

The ***salting-out*** technique is one of the most commonly used methods for protein purification from a solution, it offers a simple and rapid method. It can be used to fractionate proteins, as proteins will **precipitate** at different points with increases in **salt concentration.** Namely, **ammonium sulfate** is an ideal salt for the **fractionation of enzymes** due to its ability to maintain protein structure

the protein's structure and therefore its activity, it is highly soluble, inexpensive, low toxicity to enzymes, and low cost and serves as a preservative.

This method is based on the difference in protein solubility at **different ammonium sulfate concentration**s; in highly saline solutions, the solubility of most proteins is reduced (Burgess, 2009). Consequently, the proteins are prevented to form hydrogen bonds with water and the salt facilitates their interaction with each other forming aggregates that afterward precipitate out of solution.

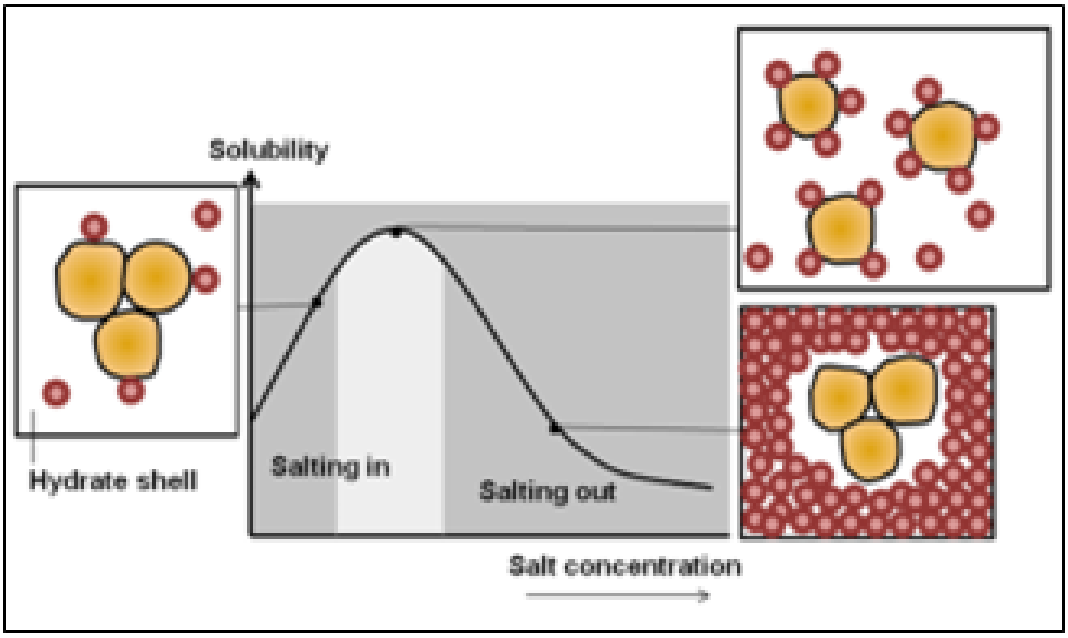


Figure 12: Salting in and salting out of proteins (Duong-Ly,2014)**.**

Salting-out technique *method*

- Thaw the enzyme sample at 4°C.
- Prepare a container filled with ice to maintain cold conditions during handling.
- Measure a volume of 100 mL of the enzyme sample.
- Weigh the required amount of ammonium sulfate to obtain a final concentration of 80%, according to the Dawson (1969) precipitation table at 4°C.
- Slowly add the ammonium sulfate to the supernatant while gently stirring on ice.
- Keep the mixture at 4°C with gentle stirring for 18 h.

Table4: final concentration of ammonium sulfate (Dawson *et al*., 1969).

Initial concentration of ammonium sulfate (percentage saturation at 0 °C)	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 100 ml of solution																
0	10.6	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	65.0	69.7
5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57.0	61.5	66.2
10	5.3	8.1	10.9	13.9	16.9	20.0	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1	62.7
15	2.6	5.4	8.2	11.1	14.1	17.2	20.4	23.7	27.1	30.6	34.3	38.1	42.0	46.0	50.3	54.7	59.2
20	0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	349	38.7	42.7	46.9	51.2	55.7
25		0	2.7	5.6	8.4	11.5	14.6	17.6	21.1	24.5	28.0	31.7	35.5	39.5	43.6	47.8	52.2
30			0	2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5	48.8
35				0	2.8	5.7	8.7	11.8	15.1	18.4	21.8	25.4	29.1	32.9	36.9	41.0	45.3
40					0	2.9	5.8	8.9	12.0	15.3	18.7	22.2	25.8	29.6	33.5	37.6	41.8
45						0	2.9	5.9	9.0	12.3	15.6	19.0	22.6	26.3	30.2	34.2	38.3
50							0	3.0	6.0	9.2	12.5	15.9	19.4	23.0	26.3	30.8	34.8
55								0	3.0	6.1	9.3	12.7	16.1	19.7	23.5	27.3	31.3
60									0	3.1	6.2	9.5	12.9	16.4	20.1	23.9	27.6
65										0	3.1	6.3	9.7	13.2	16.8	20.5	24.4
70											0	3.2	6.5	9.9	13.4	17.1	20.9
75												0	3.2	6.6	10.1	13.7	17.4
80													0	3.3	6.7	10.3	13.9
85														0	3.4	6.8	10.5
90															0	3.4	7.0
95																0	3.5
100																	0

Removal of salt molecules by dialysis

1. Introduction

α-Amylase is an important enzyme that catalyzes the breakdown of starch into simple sugars. To study its properties or use it in industrial applications, it is necessary to obtain it in a pure and active form. After ammonium sulfate precipitation, excess salt can interfere with enzymatic activity. Dialysis therefore allows the removal of these unwanted small molecules while retaining the enzyme, making it an essential step in the purification process.

2. Specific objectives

- Remove the ammonium sulfate present after fractional precipitation.
- Purify α-amylase to improve its purity and activity.

Principle

Review

Dialysis is a purification technique used to separate macromolecules, such as enzymes or proteins, from small molecules, mainly salts or other low-molecular-weight impurities. The process is based on the selective permeability of a semipermeable membrane, typically made of cellulose and containing microscopic pores. These pores allow the passage of small diffusible molecules (such as ions, salts, and buffer components) while retaining larger molecules like proteins and enzymes inside the dialysis bag (Zakowicz, 2024). The principle of dialysis relies on the phenomenon of osmosis and diffusion. When the enzyme solution is placed inside the dialysis tubing and immersed in a large volume of buffer, small molecules diffuse through the membrane from the region of higher concentration (inside the bag) to the region of lower concentration (the external buffer). Over time, an equilibrium is reached between the two compartments, reducing the concentration of unwanted small molecules in the enzyme solution (Thermo Fisher Scientific overview on buffer exchange, 2025).

To improve the efficiency of desalting, the external buffer is periodically renewed, which restores the concentration gradient and promotes further diffusion of salts and unwanted impurities. This gradual process ensures the complete removal of excess salts while maintaining

the enzyme in a stable and suitable buffer for subsequent analyses or purification steps (Creative Proteomics buffer exchange overview, 2025).

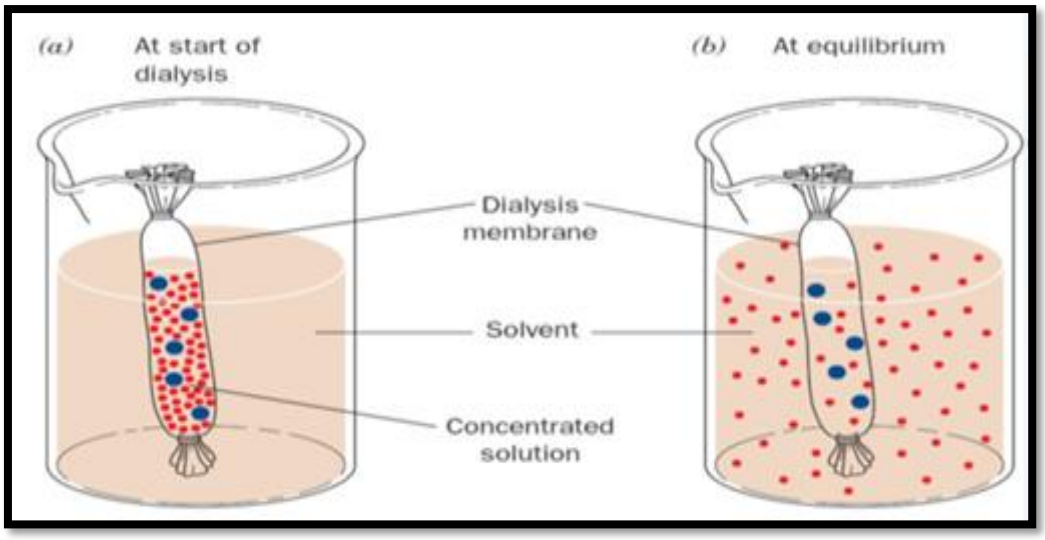


Figure 13: Principle of dialysis (Creighton, 1993).

(a): Only small molecules can diffuse through the pores in the bag, which is shown here as a tube knotted at both ends,

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

Desalinate using dialysis ***method***

a) Preparation of the dialysis tubing

1. Cut the dialysis tubing (small size) into pieces of convenient length (15 to 20 cm)
2. Incubate it in distilled water for 10 min, then in ethanol (50%) for 10min.
3. Soak the dialysis tubing in a boiling solution of 2% sodium bicarbonate and 1mM EDTA.
4. Store it in ethanol (50%) at 4°C.
5. Before using the dialysis tubing, rinse it with distilled water, then with phosphate buffer (50mM, pH=7) from the outside and the inside.

b) Dialysis

1. After incubation for 18h at 4°C with gentle agitation, centrifuge the precipitate at 4500 rpm for 30min at 4°C. The pellet is taken up in potassium phosphate buffer (50mM, pH=7).
2. Introduce the mixture into a dialysis tubing/bag.
3. Seal the tubing, using any of the standard dialysis tubing closures, to prevent leaks.

4. Maintain the tubing in a beaker, with gentle shaking to improve salt removal. at 4°C overnight against 1L of phosphate buffer (dialysis buffer: 50mM, pH=7), until the α-amylase activity is stabilized.
5. Change the dialysis buffer at least once during equilibrium.
6. Transfer the sample to a clean tube (Ghosh, 2023).

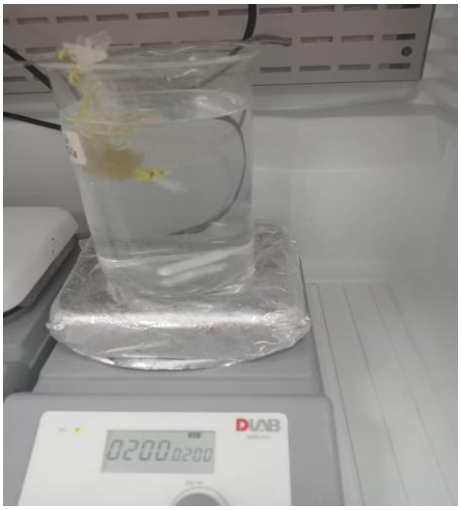


Figure14: Dialysis for Enzyme Purification (Khodja B, 2024).

Size exclusion chromatography (gel filtration)

VI

1. Introduction

After the ammonium sulfate precipitation and dialysis steps, an additional purification step is required to improve the purity of the obtained α-amylase enzyme. This purification step makes it possible to remove contaminating proteins and residual impurities while preserving enzymatic activity.

In this practical session, a chromatographic technique will be used to fractionate the proteins in the mixture and isolate α-amylase. This step represents an essential phase in the overall purification process, allowing an increase in the specific purity of the enzyme and preparing the collected fractions for subsequent analyses (enzymatic activity assay, SDS-PAGE, etc.).

2. Specific objectives

- Perform the purification of the α-amylase enzyme by size-exclusion chromatography (gel filtration).
- Collect and analyze the different fractions obtained after elution.
- Identify the fractions containing enzymatic activity.

Principle

Review

Size-exclusion chromatography, also known as gel filtration chromatography, is a separation method based on the **size of molecules**. The principle relies on the use of a gel composed of porous beads. When a mixture of molecules passes through a column filled with this gel:

- **Large molecules**, which are too big to enter the pores of the gel, remain outside the beads. They migrate quickly through the column, following the aqueous phase surrounding the gel particles. These molecules are said to be excluded.
- **Small molecules**, which can enter the pores, take a longer path through the gel and are therefore **eluted later**.

This type of chromatography allows the separation of molecules based on their size, with the largest molecules exiting the column first. (Jiménez-Díaz *et al., 1995*). Gel filtration is a special a type of liquid–solid chromatography.

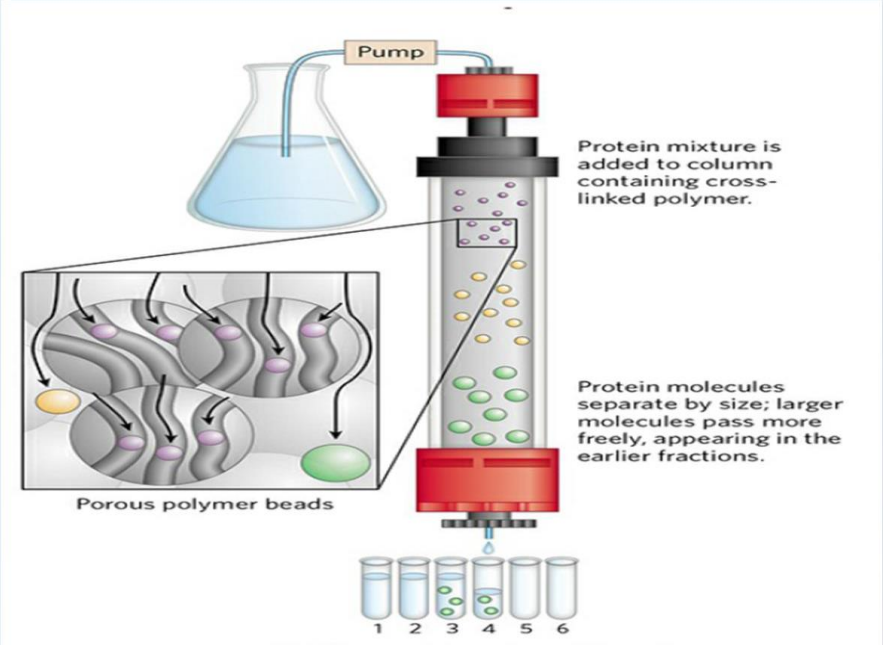


Figure 15: Principle of exclusion chromatography (Benmansour M, 2019).

Process and Preparation of the chromatography column ***method***

a) Preparation of the chromatography column

1. Rinse the chromatography column (1 x 20 cm) with distilled water.
2. Weigh out a mass of 1g of Sephadex G75.
3. Add excess potassium phosphate buffer (50mM, pH=7).
4. Incubate the mixture in a water bath at 90°C for 3 hours.
5. After swelling (Let the gel cool), stir the gel carefully using a glass rod to avoid breaking the gel beads.
6. Gradually pour the gel into the column until gelling and complete stabilization occur (up to 13 mL). Prevent the gel bed from drying by continuously adding buffer.

b) Chromatography process

1. After gelling and stabilization of the gel, introduce 2 mL of the precipitated protein sample (dialysate) into the column's walls.

2. Add potassium phosphate buffer (50 mM, pH=7).
3. Collect the α-amylase enzyme fractions (1mL) with an elution rate of 1mL/40 seconds. The entire sample will probably be eluted after about 1 hour of separation.
4. Measure the enzymatic activity of the fractions as well as the total protein concentration.
5. Mix the enzymatically active fractions in a single tube and store at -20°C.
6. 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 last parameter is calculated employing the formula below.

$$\mathbf{Y}(\%) = \frac{\textbf{total enzymatic activity recovered during the purification step (U)}}{\textbf{total initial enzyme activity}} \boldsymbol{X100}$$

Remarque

At the end of the purification steps, the specific activity is determined using the Miller and Bradford assays (see Workshop 2 on enzyme production). It is expressed in units per milligram of protein and is calculated using the following equations:

$$\boldsymbol{Z}(\textbf{enzymatic activity}) = \frac{\textbf{n } (\mu\textbf{mol})}{\textbf{t (min)}}$$

$$\boldsymbol{b}(\textbf{Concentration of catalytic activity}) = \frac{\textbf{Z(U)}}{\textbf{V(ml)}}$$

$$\boldsymbol{Zsp} = \frac{\textbf{b } (\boldsymbol{U}/\textbf{ml})}{\textbf{proteins quantity}(\textbf{mg/ml})}$$

Affinity chromatography

VII

1. Introduction

As part of the enzyme purification process, a highly selective step is required to obtain an enzyme of high purity. Affinity chromatography exploits the specific interactions between the target enzyme and a ligand immobilized on a solid matrix.

In this practical session, this technique will be used to further purify α-amylase after the preliminary fractionation steps. Non-specific proteins will be removed during the washing phases, while the enzyme will be recovered by elution. This step aims to significantly increase the purity of the enzyme while maintaining its enzymatic activity.

2. Specific objectives

- Purify α-amylase by exploiting its specific binding to a ligand immobilized on the chromatographic matrix.
- Collect and identify the fractions containing active α-amylase.
- Verify the effectiveness of the purification by assessing the enzymatic activity of the collected fractions.

Principle

Review

Affinity chromatography is a highly selective purification technique that allows the isolation of a specific protein or enzyme by exploiting its specific binding interaction with a ligand immobilized on a solid matrix. Unlike other chromatographic methods, this technique does not depend on the protein's size, shape, charge, or solubility. During the process, non-specific proteins and other impurities are removed through washing steps, while the target molecule remains bound to the ligand. The bound protein is then recovered through an elution step, which typically involves changing the pH, ionic strength, or introducing a competitive molecule to disrupt the interaction. This method is widely used in biochemistry and biotechnology because it enables high purity and high recovery of the protein while preserving its biological activity. (Zuzana Bilkova *et al*., 2025).

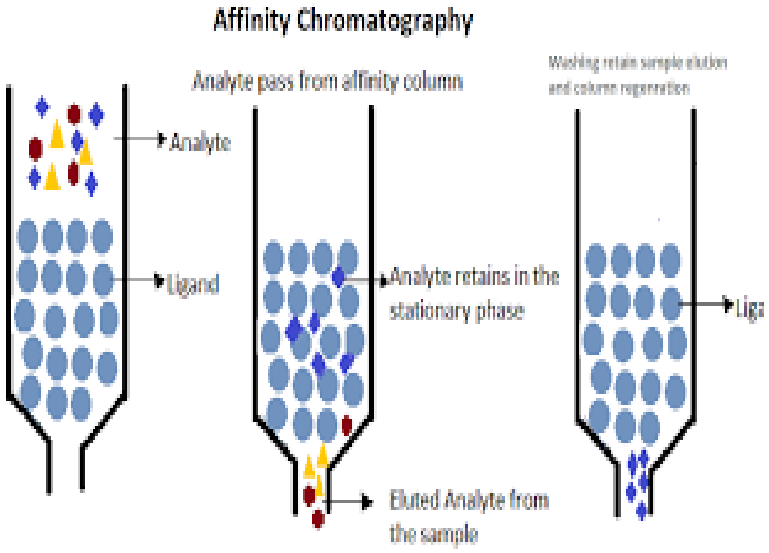


Figure 16: Concept of affinity chromatography (Thikekar, A. K.,2023).

Affinity chromatography ***method***

The protocol used for the purification of α-amylase follows the method described by Rodriguez, E. L (2020).

- Prepare the column with the appropriate matrix and equilibrate it with the binding buffer.
- Apply the previously dialyzed protein mixture onto the column to allow α-amylase to specifically bind to the immobilized ligand.
- Wash the column to remove non-specific proteins and elute the active enzyme.
- Measure the enzymatic activity of the collected fractions.

Evaluation of purification (Purification table, SDS-PAGE, zymogram)

1. Introduction

After producing, extracting, and isolating the extracellular amylolytic enzymes from *Bacillus subtilis*, the effectiveness of the purification process was evaluated through several complementary analytical techniques. Among these, SDS-PAGE was used to separate proteins based on their molecular weight, providing a visual profile of the purification steps and revealing the presence or absence of contaminating proteins. In parallel, zymography also known as in-gel assays, activity gels, or substrate gel electrophoresis was employed to detect enzymatic activity directly within the gel, allowing the identification of active amylase bands. Together, these approaches offer a comprehensive assessment of both the purity and the functional integrity of the extracted enzymes. Such combined analyses are crucial for confirming the success of the purification workflow and for further optimizing the catalytic performance of the enzymes for subsequent applications (Laemmli, 1970).

2. Specific objectives

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

3. Experimental protocols

1.1 Materials

Products	Equipments
TRIS	Micropipettes
TEMED	Centrifuge
Bis-acrylamide	Yellow pipette tips
Acrylamide	Vortex
Molecular weight protein marker(10-180)	Rocker shaker
Ice	Fume hood/ chemistry hood
Startch	Heat block (1.5mL)
Trichloroacetic acid (TCA)	Vertical electrophoresis
Lugol	Eppendorf tubes
Triton X-100	Electrophoresis generator
Methanol/butanol/isopropanol	Comb with wells
Glacial acetic acid	

3.2 Evaluation of α-amylase enzyme by purification chart/table

Principle *Review*

The evaluation of protein purification, such as that of α-amylase, using a purification table constitutes a structured and reliable method for monitoring the effectiveness of each step of the process. By systematically recording key parameters at each stage, it becomes possible to observe changes in enzyme purity throughout the procedure, identify the step at which the level of purification is maximal, and pinpoint those that may require optimization (Bekavac *et al*., 2024). This analytical approach thus highlights the overall performance of the purification strategy and, if necessary, allows the protocol to be improved.

A purification table also makes it possible to calculate several essential quantitative indicators, such as purification yield (Y), total protein content, the purification factor (or enrichment factor) (F), as well as the specific activity of the enzyme (Protein purification, 2025). These parameters provide a comprehensive overview of the efficiency of the purification process, allowing both qualitative and quantitative evaluation of the enzymatic preparation (Bekavac *et al*., 2024; Protein purification, 2025).

Method

1. Complete the table below (Table3)
2. For each purification step (from crude sample to chromatography), determine the volume, total protein content, then calculate the amylase activity, specific activity and purification yield using the **following formulas:**

$$\boldsymbol{Zsp} = \frac{\textbf{Concentration of catalytic activity}(\boldsymbol{U}/\textbf{ml})}{\textbf{proteins quantity}(\textbf{mg}/\textbf{ml})}$$

$$\boldsymbol{f} = \frac{\boldsymbol{enzymatic\ activity\ of\ each\ step\ of\ purification}\ (\textbf{U}/\textbf{mg})}{\textbf{proteins quantity}(\textbf{U}/\textbf{mg})}$$

$$\textbf{Y} = \frac{\boldsymbol{Total\ enzymatic\ activity\ recovred\ during\ the\ purification\ step}(\boldsymbol{U})}{\textbf{total initial enzyme activity}(\textbf{U})}\ \boldsymbol{X}\textbf{100}$$

Table 5: α-amylase purification chart.

Purification step	Sample volume (mL)	Total Protein content (mg)	Amylase activity (U)	Specific activity (U/mg)	Purification yield (%)
Crude sample					
Ammonium sulfate Precipitation					
Exclusion chromatography					
Affinity chromatography					

3.3 Evaluation of protein purification by SDS-PAGE (polyacrylamide gel electrophoresis)

Principle *Review*

Polyacrylamide gel electrophoresis (PAGE) is an analytical technique used to separate the components of a protein mixture under the influence of an electric field (Rath *et al.*, 2021). In its commonly used SDS-PAGE form, the separation relies exclusively on the molecular weight of the proteins. This is made possible by the presence of sodium dodecyl sulfate (SDS), a strong anionic detergent that denatures proteins and uniformly coats them with negative charges (Kurien & Scofield, 2020). As a result, differences in native charge or three-dimensional structure are eliminated, allowing proteins to migrate through the polyacrylamide matrix solely according to their size (Rath *et al.*, 2021). Smaller proteins move more rapidly through the gel pores, while larger proteins migrate more slowly. This method provides a reliable and reproducible way to analyze protein purity, estimate molecular weights, and monitor purification steps in biochemical studies (Mahmood & Yang, 2025)

Preparation of SDS-PAGE gels *method*

- Take the mold to make the gels and close the ends with the stops so that the gel does not leak.
- Make the separation gel (12%) by mixing it in the order shown in the tables below. After putting APS and TEMED into the SDS-PAGE separation gel solution, the gel will polymerize quickly, so add these two reagents when pouring.

- Pour softly separating gel, leaving 2 cm below the bottom of the comb for the stacking gel and pay attention to remove bubbles.
- Layer the top of the separating gel with isopropanol or butanol (20V butanol + 1 V distilled water, shaking, upper phase will be used). This can help remove bubbles at the top of the gel and will also prevent the polymerized gel from drying out. In 30 min, the gel should be entirely polymerized.
- Throw the isopropanol or butanol and rinse remaining traces of isopropanol with distilled water.
- Prepare the stacking gel (5%), following the same procedure as the separating gel and pour it onto the first gel (on top).
- Place the comb to form the wells.
- After polymerization, clamp/secure the gel in the device of the apparatus, and fill both buffer chambers with gel running buffer according to the instructions for the specific apparatus.

Table 6: Composition of electrophoresis gels

Products	**Separating gel 12%(8ml)**	**Stacking gel(5ml)**
Distilled water	3,4 mL	3,5Ml
40%(Acrylamide:bisacrylamide (38:2)	2,4 mL	0,75 ml
Tris	2 mL (1,5M, pH8,8)	0,65 mL (1M, pH6,8)
SDS10%	80 µL	50 µL
APS10%	80 µL	50 µL
TEMED	8 µL	5µL

Protein concentration of culture supernatant by the TCA ***method***

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

- Add 20µL of charging buffer to concentrate protein with TCA (pellet).
- Incubate in a 95°C water bath for 10 min.
- Load samples and molecular weight protein marker into the wells to separate them by electrophoresis. The separation will begin first in the stacking gel for approximately 30 min at 100V, then in the separating gel for about 90 min at 120V.
- At the end of separation on polyacrylamide gel, wash the gel with 3 aliquots of water, shaking for 5 min each, and remove all water from the gel container.
- Cover the gel completely with stain solution (add enough Coomassie stain to cover the gel) and let the gel stain for 1h on a shaker to reveal the bands present in the gel. If the protein signal is low, stain overnight.
- Pour off the Coomassie stain and rinse gels with water.
- Add fresh destain solution to cover the gel for 10 minutes on a shaker.
- Pour the destain solution onto the gel, incubate for 10 minutes to overnight, and analyze the protein bands.

Reading *Complement*

Reading of the SDS-PAGE gel and construction of the standard curve:
The interpretation of an SDS-PAGE gel is based on comparing the protein bands obtained with a molecular weight marker, which allows estimation of protein size according to their migration through the gel. As described by Nelson and Cox (2021)**,** SDS denatures proteins and imparts a uniform negative charge proportional to their length, enabling separation primarily based on molecular weight. To construct the standard curve, the migration distance of each marker band is measured and plotted against the logarithm of the molecular weight, resulting in a characteristic linear relationship **(**Hames, 2019**).** This curve is then used to determine the molecular weight of unknown proteins by interpolating their migration distance. Analysis of the gel shows that band **position** provides information about protein size, while band intensity reflects relative protein abundance; the presence of multiple bands in a single lane may indicate partial purity or protein degradation **(**Kurien & Scofield, 2020**).**

3.4 Evaluation of hydrolytic enzyme α-amylase by Zymography

Principle *Review*

The zymography is a method to assess the enzymatic activity of proteins either in situ or by separating them with electrophoresis (detection of hydrolytic activity) (He *et al.*, 2023). This electrophoretic method relies on an SDS-PAGE gel impregnated with an appropriate substrate, which is degraded by the resolved enzyme during the incubation period (Rodrigues *et al.*, 2024). Thus, the enzyme will be visualized using the substrate conversion technique and the specific stain, allowing both the identification of enzyme activity directly on the gel and an estimation of the enzyme's molecular mass by comparison with the profile obtained by SDS-PAGE (Martínez *et al.*, 2025).

Method

1. The approach follows the same standards as SDS-PAGE, with some modifications *(Hadadi and Hamrani, 2017).*

1. Prepare samples without adding reducing agents (ß-mercaptoethanol) or heat treatment.
2. Make the separation gel by adding 5mL of starch solution (1%).
3. After 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 an appropriate buffer for 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 destain is the same as SDS-PAGE.

Characterization of the α-amylase enzyme and determination of the effect of physicochemical parameters on the enzyme's activity and stability

1. Introduction

In order to characterize the amylolytic enzymes produced by the *Bacillus subtilis* strain, several tests are used, in particular solvent, detergent, pH, and temperature variation tests, to determine the optima of enzyme production and activity through enzymatic activity assays (Gupta *et al*., 2022). These parameters are essential for understanding enzyme stability, catalytic efficiency, and suitability for industrial applications, as variations in pH and temperature strongly influence the structure and function of α-amylases (Kumar *et al*., 2023). Furthermore, evaluating enzyme tolerance to solvents and detergents provides valuable insight into its robustness and potential use in harsh industrial processes such as starch processing and detergent formulation (Sundarram & Murthy, 2021; Li *et al*., 2024).

2. Specific objectives

- Determine the effect of temperature on amylolytic activity
- Measure the effect of pH on amylolytic activity
- Assess the effect of inhibitors on amylolytic activity
- Observe the effect of metal ions on amylolytic activity

3. Experimental protocols

3.1 Materials

Products	Equipments
• Starch • Calcium chloride (CaCl2) • Ferric trichloride or Iron (III) chloride (FeCl3) • Magnesium dichloride (MgCl2) • Acetone • β-mercaptoethanol • Ethylenediaminetetraacetic acid (EDTA) • DNSA reagent • Dimethyl sulfoxide (DMSO)	• Micropipettes • Vortex • pH meter • Precision balance • Spectrophotometer • Erlenmeyer flasks • Beakers • Spatulas • Bunsen burner • Pipette tips

3.2 Effect of physico-chemical parameters on enzyme activity and stability

Effect of pH on α- amylase activity and stability *method*

According to Lily *et al*. (2012**),** the following procedure was carried out:

1. Prepare potassium phosphate buffer solutions (50 mM) with pH values ranging from 4 to 10.
2. Prepare a 1% (w/v) starch solution in each phosphate buffer.
3. In each test tube, mix one volume (1V) of purified enzyme with an equal volume (1V) of buffered starch solution (V/V).
4. Incubate the reaction mixtures at 70 °C for 10 minutes.
5. Add two volumes (2V) of DNSA reagent to each tube to stop the enzymatic reaction.
6. Measure the amylolytic activity using the DNSA method.
7. Express pH stability as a percentage of residual activity, considering the initial enzyme activity at each pH as 100%.
8. Calculate the relative enzyme activity using the following equation:

$$\textbf{\textit{Relative Enzyme Activity}}\ (\%) = \frac{\textbf{\textit{residual activity}}}{\textbf{initial enzyme activity}} \textbf{\textit{x}}\mathbf{100}$$

Effect of temperature on α-amylase activity and stability *method*

- Prepare a 50 mM potassium phosphate buffer at pH 7.
- Prepare a 1% (w/v) starch solution in the buffer.
- Mix one volume (1V) of purified enzyme with an equal volume (1V) of the buffered starch solution (V/V) at the optimum pH.
- Incubate the reaction mixture at different temperatures ranging from 30°C to 100°C for 10 minutes.
- Measure the enzyme activity at each temperature to determine the optimal temperature.
- To study thermostability, incubate the enzyme at 70°C, 80°C, and 90°C for 3 hours.
- Take samples at 30-minute intervals and immediately cool them on ice.
- Determine the residual enzymatic activity of each sample under optimal assay conditions.

- Consider the untreated enzyme as the control (100%) and express thermostability as the percentage of residual activity relative to the control.

3.3 Effect of solvent on α-amylase activity and stability

 *method*

- Pre-incubate the enzyme-starch buffer in 10% acetone or DMSO for 10 min at optimal temperature.
- After incubation, measure amylolytic activity.
- Continue incubation in the presence of the solvent.
- Measure the activity after 2 hours to test stability (*Lily et al., 2012*).

3.4 Effect of inhibitors on α-amylase activity and stability

 *method*

- 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, inhibitor) or β-mercaptoethanol (10 mM) for 10 minutes at the optimal temperature.
- Measure the amylolytic activity after 10 minutes.
- Continue incubation in the presence of the solvent for 2 hours.
- Measure the activity after 2 hours to test stability (Kherouf, 2022).

3.5 Effect of metal ions on α-amylase activity

 *method*

- Perform 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 50 mM phosphate buffer.
- Add 1 % starch and measure the relative enzyme activity under standard assay conditions. (Lily *et al.*, 2012).

Determination of the enzyme kinetic parameters

1. Introduction

Enzyme kinetics studies the rate of enzyme-catalyzed reactions as a function of substrate concentration. It allows the characterization of enzymatic activity through essential kinetic parameters, notably the maximum reaction activity (Vmax) and the Michaelis–Menten constant (Km), which describe the efficiency and affinity of an enzyme for its substrate (Michaelis & Menten, 1913). Determining these parameters contributes to a better understanding of enzyme function and catalytic behavior and is fundamental in biochemical studies (Nelson & Cox, 2021).

2. Specific objectives

- Evaluate the kinetic parameters (Km, V max, and Vi).

3. Experimental protocols

3.1 Materials

Products	**Equipments**
Starch Potassium phosphate buffer (50 Mm) DNSA reagent	Micropipettes Spectrophotometer Tubes Pipette tips Micropipettes Water bath

Principle *Review*

The principle of this experiment is based on measuring the initial reaction activity at different substrate concentrations (Johnson & Goody, 2021). The obtained activity is analyzed using the Michaelis–Menten model to determine the kinetic parameters Vmax and Km (Cornish-Bowden, 2022)

In addition, the Lineweaver–Burk representation, which consists of plotting the inverse of the reaction rate (1/v) versus the inverse of the substrate concentration (1/[S]),

is used to obtain a linear relationship and facilitate the determination of these kinetic parameters (Segel, 2023). The graphical analysis allows interpretation of the kinetic behavior of the studied enzyme (Fersht, 2024).

3.2 Proceding & reading  *method*

- Prepare starch concentrations of 0.2 to 1mg/mL in phosphate buffer (starch buffered).
- Preheat starch buffer to 70°C.
- Mix 1V supernatant with 1V buffered starch (V/V).
- Incubate tubes at 50°C/ 37°C at 5min.
- Stop the reaction by adding 2V DNSA to each tube.
- Incubate samples in a water bath at 100°C for 5 min.
- Measure the amount of reduced DNSA at 540 nm.
- From the curve, determine the kinetic parameters Km and Vmax of amylase.

Evaluation, Debate and closing day

1. **Evaluation:**

Exercise 1:

For each question (Q1 to Q8), check the correct answer(s) among options A, B, C, and D in the table below.

Q1 – During the assay of α-amylase activity using the DNS method:

A) DNS reacts with reducing sugars

B) Absorbance is measured at 595 nm

C) The higher the starch concentration, the more the signal is always linear

D) Maltose can be used as a standard

Q2 – Regarding Km, identify the correct statement:

A) It directly measures the enzyme's affinity for its substrate

B) It represents a reaction constant for which [S] = Vmax / 2

C) It is the same constant for all enzymes

D) It depends on the temperature and pH of the reaction

Q3 – Why must the Sephadex gel be rehydrated before use?

A) To increase the solubility of the gel

B) To allow the gel to swell and stabilize

C) To remove impurities

D) To adjust the gel's pH

Q4 – The stacking gel is used to:

A) Separate proteins according to size

B) Concentrate proteins into a thin band

C) Improve resolution

D) Facilitate uniform migration

Q5 – The principle of the Bradford method is based on:

A) Interaction of the dye with basic amino acids

B) Reaction with peptide bonds

C) Color change brown → blue

D) Oxidation of proteins

Q6 – What mass of NaOH is needed to prepare 250 mL of a 2.0 M solution?

A) 10 g

B) 20 g

C) 40 g

D) 80 g

Q7 – During the study of α-amylase activity, why must the reaction mixture be incubated?

A) To allow the enzyme to denature quickly

B) To favor the enzymatic reaction and product formation

C) To change the pH of the substrate

D) To directly measure the enzyme concentration

Q8 – Why is enzymatic purification often carried out at low temperature?

A) To increase the reaction rate

B) To prevent bacterial contamination

C) To preserve enzymatic activity

D) To precipitate proteins

	A	B	C	D
Q1				
Q2				
Q3				
Q4				
Q5				
Q6				
Q7				
Q8				

Exercise 2:

α-amylase is an enzyme widely used in several industrial sectors, including food, pharmaceutical, and biotechnological industries. Its large-scale production relies on processes involving microorganisms and controlled fermentation technologies.

Questions :

1. Describe the production process of α-amylase from a microorganism (bacterial or fungal).
2. What is the importance of optimizing the culture medium for enzyme production?
3. What are the critical parameters to control during fermentation?

Exercise 3:

After α-amylase is produced by microbial fermentation, the enzyme is obtained in crude form, mixed with other proteins, metabolites, and salts from the culture medium. To study or use it for industrial purposes, appropriate purification steps are necessary, such as ammonium sulfate precipitation, usually followed by dialysis.

Questions:

1. Explain the principle of ammonium sulfate precipitation.
2. If using 0–40% saturation followed by 40–70%, what does each fraction represent?
3. Why is dialysis performed after precipitation?
4. What problems may arise if dialysis is poorly conducted?

Exercice 4:

The table below shows the evolution of total protein content and α-amylase activity during different purification steps, from crude extract to size-exclusion chromatography.

Step	**Protéins (mg)**	**Total activity (U)**
Crud extract	800	9600
Précipitation (30–60 %)	200	7200
Dialysis	180	7500
Size- exclusion Chromatography	60	5400

Questions:

1. Calculate the specific activity.
2. Identify any anomalies in these results.
3. Propose a possible experimental explanation.
4. How can the optimal pH and optimal temperature for α-amylase activity be determined?

Exercice 5:

To assess the purification of a protein, different methods can be used, such as quantitative measurements of enzymatic activity or protein analysis techniques.

Questions:

1. Calculate the specific activity.
2. Identify any anomalies in these results.
3. Propose à possible experimental explanation.
4. How can the optimal pH and temperature for α-amylase activity be determined?

2. Debate and closing day

At the end of the workshop, a discussion session is planned to:

- Evaluate the results obtained and compare the different methods for purification and assessment of α-amylase.
- Discuss the effects of physicochemical parameters on the enzyme's activity and stability, and the implications for industrial and biotechnological applications.
- Identify critical points and possible sources of experimental errors.
- Answer participants' questions and consolidate the knowledge gained on enzyme purification and characterization techniques.

The workshop concludes with a summary of the objectives achieved, submission of the final report, and recommendations for future practical work, in order to reinforce the understanding of enzymatic engineering concepts.

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Appendix

Use of Artificial Intelligence Tools

During the preparation of this document, artificial intelligence tools, including ChatGPT, Quillbot, Gemini, and DeepL, were employed strictly for editorial and linguistic purposes. These tools helped optimize sentence formulation, the coherence of the argumentation, as well as the overall readability and structure of the text.

It should be noted that the use of these technologies did not in any way affect the scientific content, experimental data, or the analyses and conclusions presented. Their role was limited to stylistic and organizational support, ensuring a final document that is clear, precise, and consistent with academic and scientific standards.