الجمهورية الجزائرية الديمقراطية الشعبية وزارة التعليم العالي والبحث العلمي

PEOPLE'S DEMOCRATIC REPUBLIC OF ALGERIA
MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH

Academic Year 2025/2026

Higher School of Biological Sciences of Oran (ESSB d'Oran)

Department of Second Cycle

Educational Handout

Subject

Valorization of Aquatic Bioresources

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

Directed by:

Dr. ASFOURI Nadia Yasmine

Subject taught during the academic years : 2023/2024
2024/2025

Table des matières

List of Abbreviations

°C : degree Celsius

ARNr : ribosomal ribonucleic acid (rRNA)

ml : milliliter

mM : millimolar

NaOH : sodium hydroxide

tr/mi : turns per minute

NH_2 : aminyl radical

tr/min : revolutions per minute (rpm)

U/ml : units per milliliter

Foreword

This course material provides a concise overview of the practical workshops within the *Aquatic Bioresources Valorization* module, intended for second-year postgraduate students specializing in Enzymatic Engineering at the Higher School of Biological Sciences, Oran.

The practical workshops on aquatic bioresources valorization constitute a strategic framework for strengthening scientific and technical competencies in the processing and sustainable utilization of marine and freshwater organisms. The training focuses on advanced methodologies for fractionation and extraction of high-value biomolecules (proteins, lipids, pigments, polysaccharides), as well as on the development of innovative bioproducts with strong industrial potential.

In addition, these workshops provide a basis for evaluating the economic and ecological potential of aquatic bioresources through the integration of circular bioeconomy approaches. They also aim to promote synergies among academic researchers, industrial partners, and institutional stakeholders, with the overarching objective of optimizing valorization pathways while ensuring the preservation and sustainability of aquatic ecosystems.

Course Objectives

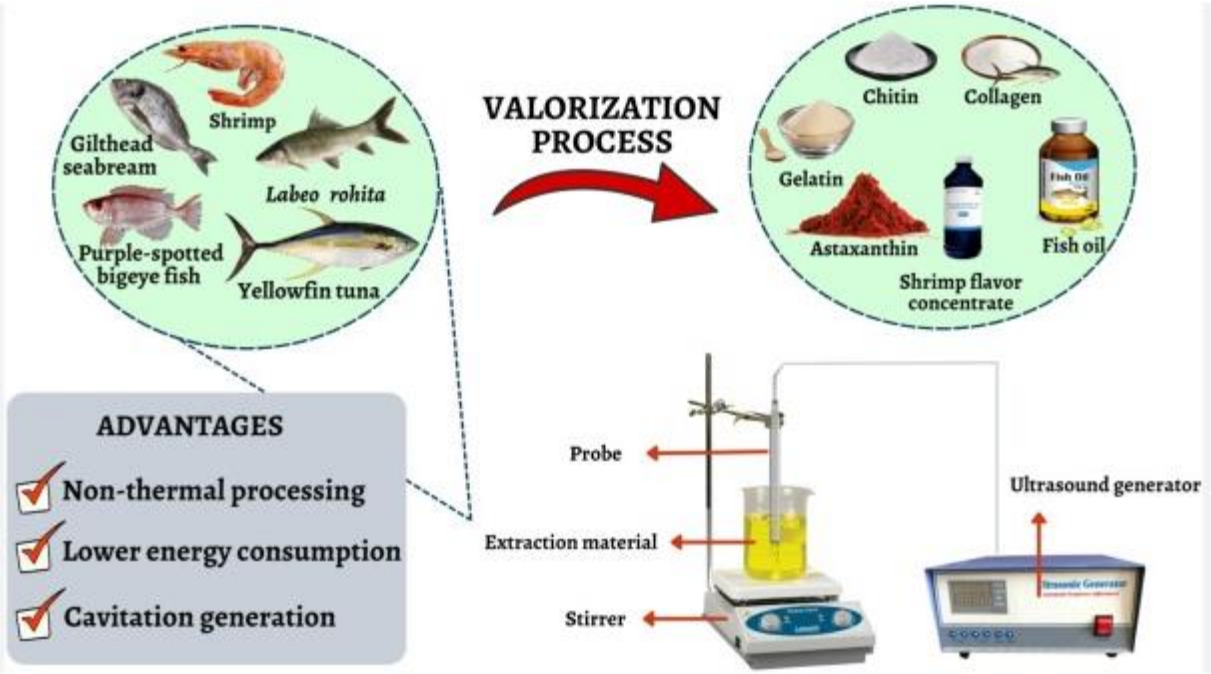
- Strengthen practical skills in biotechnological processes for the transformation and valorization of aquatic bioresources (enzymes, peptides, biomaterials).
- Master advanced analytical techniques for the characterization of biomolecules (SDS-PAGE, enzymatic assays, evaluation of biofunctional activities).
- Valorize marine co-products and crustacean by-products by converting them into high value-added products (chitosan, hydrolysates, microcapsules).
- Explore the bioactive potential of marine extracts, particularly their antioxidant and antimicrobial properties, for applications in health, nutrition, and cosmetics.
- Promote an integrated circular bioeconomy approach applied to aquatic bioresources, combining innovation, sustainability, and industrial performance.

Recommended Prerequisites

For the successful completion of the workshops on aquatic bioresources valorization, students are expected to possess the following prerequisites :

- **Foundations in Microbiology**: knowledge of microbial culture techniques, growth conditions, sterilization methods, and aseptic handling.
- **Basic Concepts in Biochemistry**: understanding of the structure and function of proteins, enzymes, and peptides, as well as the principles of enzymatic hydrolysis.
- **Core Knowledge in Biotechnology**: familiarity with fermentation processes, extraction, purification, and transformation of biomolecules.
- **Skills in Biochemical Analytical Techniques**: use of spectrophotometry, protein quantification, SDS-PAGE electrophoresis, and interpretation of experimental results.
- **Awareness of Biological Waste Valorization**: understanding of the challenges associated with the circular bioeconomy and the transformation of aquatic co-products.

Workshops Overview



Workshop 1 : Enzyme Production

This workshop is dedicated to the production and analysis of various enzymes of industrial interest. It begins with the production of subtilisin from *Bacillus subtilis*, followed by the production of chitinase from *Aspergillus niger*. The obtained enzymes are subsequently subjected to quantitative assays in order to determine their respective enzymatic activities.

The produced proteins are analyzed by SDS-PAGE gel electrophoresis, which enables the verification of their molecular profiles and purity. In addition, a zymogram is performed to directly reveal the specific enzymatic activities on the gel. This combined approach establishes a link between enzyme production, activity, and biochemical properties.

The workshop thereby provides practical training in fundamental microbiological and biochemical techniques, with a focus on determining the optimal conditions of enzymatic activity and stability (temperature and pH).

Workshop 2 : Biopeptide Production

This workshop is devoted to the production and characterization of biopeptides derived from protein hydrolysates. It begins with the preparation of hydrolysates, obtained through the controlled action of specific enzymes on selected protein sources. This step aims to release bioactive peptides with potential nutritional, functional, or therapeutic properties.

The resulting hydrolysates are subsequently subjected to physicochemical characterization in order to determine their properties, such as solubility, amino acid composition, and molecular size distribution. This approach makes it possible to establish a relationship between hydrolysis conditions and the final characteristics of the biopeptides.

The workshop thus highlights the crucial role of enzymatic processes in the valorization of protein resources.

Workshop 3 : Valorization of Crustacean By-products

This workshop focuses on the valorization of crustacean by-products through the conversion of their constituents into high value-added products. It begins with the extraction of chitin from shells, followed by its conversion into chitosan through deacetylation. The obtained chitosan is subsequently employed in the development of hydrogels and microcapsules, materials with diverse applications in the biomedical, pharmaceutical, and environmental fields.

The workshop also includes the production of chito-oligosaccharides, compounds that exhibit noteworthy biological and functional properties. This process exemplifies the application of green chemistry and biotechnological approaches for the sustainable valorization of marine co-products.

Workshop 4 : Functional Activities of Fish and Crustacean By-product Hydrolysates

This workshop is dedicated to the evaluation of the functional activities of hydrolysates obtained from fish and crustacean by-products. The extracts are subjected to antioxidant activity assays to assess their capacity to scavenge free radicals and prevent oxidative processes. In parallel, their antibacterial potential is tested against various microbial strains of sanitary relevance in order to identify inhibitory effects.

These analyses make it possible to establish a relationship between the biochemical composition of the hydrolysates and their biological properties. The workshop thus highlights the role of marine resource valorization processes in the production of bioactive compounds with

Laboratory Safety Guidelines

Prior to engaging in any experimental activity, it is essential that students fully understand and strictly comply with the following laboratory safety regulations:

- **Familiarization with safety equipment**: Be aware of the location and proper use of fire extinguishers, eye wash stations, and emergency showers.
- **Hygiene practices**: Eating, drinking, or applying cosmetics in the laboratory is strictly prohibited. Hands must be thoroughly washed before leaving the laboratory.
- **Personal protective equipment (PPE)**: Always wear appropriate PPE, including a laboratory coat, gloves, safety goggles, and closed-toe shoes.
- **Supervision and collaboration**: Never work alone in the laboratory. Inform a colleague or supervisor of your presence and planned activities before starting any experiment.
- **Chemical and equipment handling**: Handle all chemicals and instruments with caution. Do not taste, touch, or inhale substances unless explicitly authorized.
- **Labeling and waste disposal**: Clearly label all containers and samples. Dispose of waste and hazardous materials strictly in accordance with the instructor's or institution's guidelines.
- **Workstation maintenance**: Keep your workspace clean and well organized. Immediately report any spills, accidents, or injuries to the instructor or laboratory supervisor.
- **Authorization of experiments**: Conduct only authorized experiments. Do not modify equipment or carry out procedures without proper training and prior approval.

Introduction

Aquatic bioresources, encompassing algae, fish, shellfish, crustaceans, and a wide variety of by-products from fisheries and aquaculture, represent an immense reservoir of compounds with high biotechnological and industrial potential (Rai *et al.*, 2017). Their valorization refers to the transformation of these raw materials and residues into value-added products such as functional foods, bioactive peptides, bioplastics, pharmaceuticals, cosmetics, and biofuels. This approach is strongly aligned with the principles of the circular economy, aiming to reduce waste, enhance resource efficiency, and mitigate environmental impacts.

Globally, aquatic production is rapidly increasing. According to recent FAO reports, aquaculture output reached more than 87 million tonnes in 2024, with an estimated economic value exceeding USD 280 billion. However, nearly 30–40% of this biomass is generated as waste or by-products during processing, which highlights the importance of innovative valorization strategies (FAO, 2024). Current research emphasizes the extraction of high-value molecules such as proteins, lipids, polysaccharides, and pigments from aquatic organisms, as well as the conversion of processing residues into hydrolysates, biofertilizers, and sustainable feed ingredients.

Recent advances include integrated aquaculture systems combined with microalgae cultivation for nutrient recovery and CO_2 sequestration, algal biorefineries producing both biofuels and high-value metabolites, and enzymatic hydrolysis of fish waste to obtain bioactive peptides with applications in food and health. These developments not only contribute to environmental sustainability but also open new economic opportunities, particularly in the fields of biotechnology, food technology, and green chemistry (Nelson & Cox, 2021).

Despite these promising perspectives, challenges remain regarding economic feasibility, scalability, and regulatory compliance for food and pharmaceutical applications. Nevertheless, the valorization of aquatic bioresources is emerging as a strategic field at the intersection of biotechnology, environmental protection, and sustainable development, offering a fertile ground for enzymatic engineering to design eco-friendly processes and unlock new high-value applications (Theysgeur *et al.*, 2021).

1. Workshop 1 : Enzyme Production

1.1.Protease Enzymes

- **Definition of Enzymes**

Enzymes are biological macromolecules, predominantly proteins, that act as highly specific and efficient biocatalysts in living systems. Their catalytic function relies on the reduction of the activation energy barrier of biochemical reactions, thereby increasing reaction rates by several orders of magnitude compared to the uncatalyzed processes, while remaining structurally unaltered upon completion of the catalytic cycle (Nelson & Cox, 2021).

Enzymatic specificity arises from the stereoelectronic and chemical complementarity between the active site and its cognate substrate, resulting in the transient formation of an enzyme–substrate complex and subsequent stabilization of the transition state (Berg *et al.*, 2019).

The activity of most enzymes is restricted to narrow physiological conditions of temperature, pH, and ionic strength. Their function is subject to fine-tuned regulation through allosteric modulation, covalent modification, or transcriptional and translational control of gene expression (Voet *et al.*, 2016).

In addition to protein enzymes, a distinct class of catalytic molecules known as ribozymes consists of RNA with intrinsic enzymatic activity, demonstrating that catalysis is not exclusively a protein-based phenomenon (Doudna & Cech, 2002).

Enzymes occupy a central role in cellular metabolism, signal transduction, and homeostatic regulation. Beyond their biological relevance, they represent indispensable tools in biotechnology, with applications spanning industrial biocatalysis, pharmaceutical development, food processing, and biomolecular engineering.

- **Classification of Enzymes (IUBMB)**

Enzymes are classified by the International Union of Biochemistry and Molecular Biology (IUBMB) into six major classes (EC 1 to EC 6), according to the type of reaction they catalyze.

1. Oxidoreductases (EC 1)

Oxidoreductases catalyze oxidation–reduction reactions by transferring electrons from a donor to an acceptor molecule. They are central to energy metabolism, such as cellular respiration and photosynthesis. An example is lactate dehydrogenase, which catalyzes the conversion of lactate to pyruvate (Bisswanger, 2017).

2. Transferases (EC 2)

Transferases catalyze the transfer of functional groups (phosphate, methyl, glycosyl, or acyl groups) between molecules. They are involved in key pathways such as transamination, phosphorylation, and glycosylation. For instance, alanine aminotransferase transfers an amino group from alanine to α-ketoglutarate, producing glutamate and pyruvate.

3. Hydrolases (EC 3)

Hydrolases catalyze the cleavage of chemical bonds through the addition of water. They are major actors in the degradation of proteins, polysaccharides, and lipids. Proteases (EC 3.4), such as subtilisin, hydrolyze peptide bonds, while lipases hydrolyze triglycerides. Hydrolases are widely exploited in the food, detergent, and pharmaceutical industries (Caruso *et al.*, 2020).

4. Lyases (EC 4)

Lyases catalyze the breaking of C–C, C–O, C–N, or other bonds without hydrolysis or oxidation, often generating a double bond or a new functional group. A well-known example is aldolase, which cleaves fructose-1,6-bisphosphate into two trioses during glycolysis (Bisswanger, 2017).

5. Isomerases (EC 5)

Isomerases catalyze intramolecular rearrangements, producing isomers without altering the molecular formula. For example, phosphoglucose isomerase converts glucose-6-phosphate into fructose-6-phosphate in glycolysis (Caruso *et al*, 2020).

6. Ligases (EC 6)

Ligases catalyze the covalent joining of two molecules, usually coupled with the hydrolysis of ATP. DNA ligase, for example, seals breaks in DNA strands by forming phosphodiester bonds. Ligases are essential tools in molecular biotechnology, particularly in DNA cloning and genetic engineering.

- **Workshop Objective**

The aim of this workshop is to provide hands-on training on the main methods for producing and/or extracting enzymes of interest, with a particular focus on proteases. Special emphasis will be placed on applications in the valorization of aquatic bioresources, highlighting the role of enzymes in the conversion of marine biomolecules into value-added products (e.g., bioactive peptides, chitin derivatives, marine lipids).

1.2.Materials and Methods

1.2.1. Biological material

The bacterial strain used for protease production in this study was *Bacillus mojavensis* FP2, isolated from Daya Morsli Lake (Petit Lac, Oran, Algeria) on September 30, 2013, from soil attached to flamingo feathers. The strain was taxonomically identified by 16S rRNA gene sequencing, and the sequence was deposited in the GenBank database (NCBI) under accession number KR262719.

- **Revival of *Bacillus mojavensis* FP2**

The *Bacillus mojavensis* FP2 strain, preserved in glycerol at –20 °C, was revived by inoculation into Luria–Bertani (LB) broth and incubated for 24 h at 37 °C. The culture was then streaked onto inclined solid LB medium and stored at 4 °C for use throughout the experimental study.

Table 01 : Composition of Luria Bertani medium

Ingredient	Quantity (g/L)
NaCl	10
Tryptone	10
Yeast extract	5
Agar *	15

* : No agar was added in Luria Bertani broth

1.2.2. Production of subtilisin protease

200 µL of fresh culture of Bacillus mojavensis FP2 was inoculated into 50 ml of optimal production medium and then incubated at 50 °C for 24 hours. The culture was centrifuged at 4500 rpm for 20 minutes at 4 °C, and the supernatant was collected and stored at -20 °C.

Table 02 : Protease production medium

Ingredient	Quantity (g/L)
Casein	5
Yeast extract	4
MgCl	0.5
K2HPO4	0.5
KH2PO4	0.5

1.2.3. Enzymatic Activity Assay of Protease

The enzymatic activity of the protease was measured using the Anson method (1938) (Anson, 1938) with casein as the substrate. The assay was performed by mixing 5 ml of 50 mM Glycine-NaOH buffer at pH 10 containing 0.65% casein with 1 ml of the enzyme preparation, followed by incubation at 50 °C for 5 minutes. The reaction was stopped by adding 5 ml of 110 mM trichloroacetic acid. The mixture was then incubated at room temperature for 30 minutes and centrifuged at 10,000 rpm for 5 minutes at 4 °C. Next, 2 ml of the supernatant was mixed with 5 ml of 0.5 M Na2CO3 and 1 ml of 0.5 M Folin–Ciocalteu reagent. After incubation in the dark at room temperature for 30 minutes, absorbance was measured at 660 nm using a spectrophotometer.

A calibration curve was constructed using tyrosine solutions ranging from 0 to 50 mg/ml.

Enzymatic activity is expressed in units/ml, corresponding to the amount of enzyme catalyzing the conversion of 1 µmol of substrate per minute.

1.2.4. Optimal Conditions for Enzyme Activity and Stability (Temperature and pH)

- **Effect of Reaction Time on Protease Activity**

  To determine the optimal reaction time of subtilisin protease from Bacillus mojavensis FP2, enzymatic activity was measured every 5 minutes over a period of 30 minutes at pH 10 and 50 °C.

- **Effect of pH on Protease Activity**

  The effect of pH on the activity of subtilisin protease from Bacillus mojavensis FP2 was investigated. Protease activity was measured at 50 °C for 5 minutes at different pH values ranging from 7.0 to 12.0 using the following buffers : 50 mM Glycine-NaOH for pH 9.0 to 12.0; 50 mM Tris-HCl for pH 8.0; and 50 mM potassium phosphate for pH 7.0.

- **Effect of Temperature on Protease Activity**

  The effect of temperature on subtilisin protease enzymatic activity was studied by measuring activity at various temperatures (from 37 to 70 °C) for 5 minutes at pH 10.0.

1.2.5. Detection of Chitinolytic Activity

The detection of chitinolytic activity consists of inoculating actinomycete strains by simple streaking on a minimal agar medium supplemented with colloidal chitin [CCA medium (g/L) :

Table 03 : Composition of CCA medium

Ingredient	Quantity (g/L)
$ZnSO_4$	0.001
$FeSO_4 \cdot 7H_2O$	0.01
$MgSO_4$ $5H_2O$	0.5
K2HPO4	0.7
KH2PO4	0.3
$MnCl_2$	0.001
Colloidal chitin	20
Agar	20

After 8 days of incubation, the production of extracellular chitinase is evidenced by the appearance of clear zones around the colonies. The diameter of the hydrolysis zones is measured for each strain.

To select the most efficient strains, the detection is first carried out on CCA medium adjusted to three different pH values (5, 7, and 9). After incubation at 30 °C for 8 days, the strains showing the largest hydrolysis diameters are retained to repeat the test under the same conditions, but with incubation at different temperatures (20 to 50 °C). The strains that exhibit the largest hydrolysis diameters, depending on pH and temperature, are selected for further study.

- **Preparation of Colloidal Chitin**

The preparation of colloidal chitin was carried out according to the modified protocol of Hsu and Lockwood (Lockwood and Hsu, 1975). For this purpose, crab shell fragments previously washed with distilled water were manually ground using a mortar and pestle for 10 minutes. The resulting powder was sieved to remove larger particles.

To dissolve the calcium compounds contained in the shell, twenty grams of this powder were treated with 150 mL of concentrated HCl (~12 M) in a 1 L glass beaker. The hydrochloric acid was added slowly, with continuous stirring for 60 minutes under a chemical hood at room temperature (25 °C).

The chitin-HCl mixture was then passed through eight layers of gauze to remove undissolved large particles. The clear filtrate obtained (~100 mL) was poured into two liters of cold distilled water to allow the precipitation of colloidal chitin. The mixture was stored at 4 °C under static conditions to promote precipitation.

After 12 h, the excess water was decanted, and the precipitate was passed through Whatman No. 3 filter paper. The retained filtrate in the paper was then thoroughly washed with distilled water (~5 L) until neutralization of the pH (pH ≈ 7). The colloidal chitin thus obtained was dried to remove maximum moisture, then sterilized and stored at 4 °C.

1.2.6. Chitinase Production

A spore suspension of the selected actinomycete strain was prepared from a culture grown on ISP_2 medium. Ten milliliters of physiological water were aseptically poured onto the plate, and the spores were scraped off using a platinum loop. The resulting suspension was then transferred into a sterile test tube, and the optical density at 620 nm (OD_{620}) was adjusted to 0.1, corresponding to a suspension of 10^7 spores/mL (Narayana and Vijayalakshmi, 2009).

The recommended culture medium for chitinase production in liquid culture is the CYS medium (g/L) :

Table 04 : Composition of CYS medium (Ph7)

Ingredient	Quantity (g/L)
yeast extract	0.5
$FeSO_4 \cdot 7H_2O$	0.1
$MgSO_4$ $5H_2O$	1
K2HPO4	2
Colloidal chitin	5

1.2.7. SDS-PAGE Electrophoretic Profiles

An SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) method was used to identify the protein profile, following the modified protocol described by Arumugam et *al*. (2018).

- **Polyacrylamide Gels**

Two types of gels were prepared for SDS-PAGE : an 8% separating gel and a 5% stacking gel. Each gel was prepared with the necessary components, poured between glass plates, and polymerized at room temperature before inserting the combs for the loading wells

- **Sample Preparation**

Collagen samples (ASC or PSC) were mixed with a loading buffer prepared by combining 250 µL of 25% glycine, 200 µL of 20% SDS, 50 µL of 5% β-mercaptoethanol, and 1 mg of

bromophenol blue (final concentration 0.1%), topped up to 1 mL with distilled water. The mixture was heated at 95 °C for 3 minutes to denature the proteins.

- **Electrophoresis**

Once fully polymerized, the gels were placed in an electrophoresis tank containing running buffer. The prepared samples, along with a 180 kDa molecular weight marker, were loaded into the wells. Electrophoresis was performed at a constant current of 20 mA per gel for approximately 1 hour and 30 minutes.

- **Gel Staining and Destaining**

At the end of the electrophoresis run, the gels were fixed and then stained with Coomassie Brilliant Blue R-250. Destaining was gradually performed in a solution composed of methanol, acetic acid, and water until the protein bands became clearly visible.

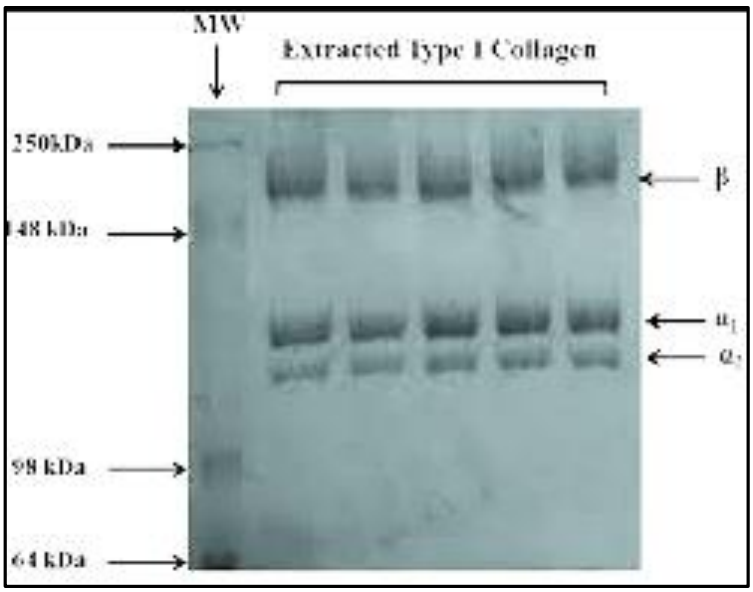


Figure 01 : SDS-PAGE profile

- **Evaluation of Chapter One**

What are the common methods used to extract proteases from microorganisms ?

What is the primary source of proteolytic enzymes ?

- **The answer key**

Microbial proteases are typically obtained from bacterial or fungal cultures produced through submerged fermentation (SmF) or solid-state fermentation (SSF). After microbial growth, extracellular enzymes are recovered by filtration or centrifugation, then concentrated using ammonium sulfate precipitation, dialysis, or ultrafiltration, and further purified through chromatographic techniques. For intracellular enzymes, a prior cell-disruption step such as sonication, mechanical grinding, or freeze–thaw cycles is required. Microorganisms represent the primary source of industrial proteases because they offer high yields, rapid production, and enzymes with excellent stability and functional diversity.

2. Workshop 2 : Production of Biopeptides

A protein hydrolysate is a product obtained by the partial or complete breakdown of proteins into smaller peptides and free amino acids, typically using enzymes (enzymatic hydrolysis), acids, or bases. This process cleaves peptide bonds, enhancing the solubility, digestibility, and bioavailability of proteins. Protein hydrolysates often exhibit specific functional and biological properties, such as antioxidant, antimicrobial, or antihypertensive activities, and are widely used in the food, nutraceutical, pharmaceutical, and cosmetic industries.

From a scientific perspective, their production not only allows the valorization of protein resources (fish, shellfish, legumes, whey, etc.) but also enables the generation of high-value biopeptides (Korhonen & Pihlanto, 2006 ; Chalamaiah *et al*., 2012).

2.1. Production of Protein Hydrolysates

The production of protein hydrolysates can be carried out by several methods, each with advantages and limitations depending on the nature of the raw material and the intended use of the product, including chemical hydrolysis, enzymatic hydrolysis, and fermentation hydrolysis.

- **Chemical Method**

  Chemical hydrolysis relies on the use of strong acidic or alkaline agents to break peptide bonds. Acid hydrolysis, typically performed with concentrated hydrochloric acid at high temperatures (110–120 °C), allows complete degradation of proteins but can destroy certain essential amino acids such as tryptophan. Alkaline hydrolysis, using sodium hydroxide or potassium hydroxide, is less common because it may cause racemization of amino acids and formation of undesirable compounds (Kristinsson & Rasco, 2000). Although faster and less expensive, this method is generally reserved for industrial applications where fine functional properties of peptides are not a priority.

- **Enzymatic Hydrolysis**

  Protein hydrolysis using exogenous enzymes, or enzymatic hydrolysis, allows better control over the process and the resulting product. The specificity of proteases influences the size, quantity, amino acid composition, and peptide profile.

Consequently, enzymatic hydrolysis is considered the most effective method for obtaining protein hydrolysates with bioactive properties (Zamora-Sillero *et al*., 2018). The enzymes used for hydrolysis may originate from animal sources (e.g., pepsin), plant sources (e.g., bromelain), or microbial sources (e.g., Alcalase, Neutrase, or Flavourzyme). Microbial enzymes are the most commonly used due to their high stability across varying pH levels and temperatures (Petrova *et al*., 2018). Enzymatic hydrolysis is preferred over microbial fermentation for producing bioactive peptides because of its shorter reaction time, ease of industrial scale-up, and predictable outcomes.

- **Fermentation Method**

Fermentation is an indirect biological approach for producing protein hydrolysates, relying on the natural enzymatic activity of microorganisms such as lactic acid bacteria (*Lactobacillus* sp.), certain yeasts (*Saccharomyces cerevisiae*), or filamentous fungi (*Aspergillus* sp., *Rhizopus* spp.). During fermentation, these microorganisms secrete a set of endogenous proteases capable of breaking down proteins into peptides and free amino acids. This method has the advantage of not requiring the external addition of purified enzymes, while also generating additional bioactive compounds such as organic acids, vitamins, and aromatic compounds (Zhao *et al*., 2016). Depending on the microbial strain employed and the culture conditions (pH, temperature, duration, substrate), the peptide profile and functional properties of the hydrolysate can be modulated. This technique is widely used in the valorization of marine by-products and in the production of functional ingredients for food, nutraceutical, and cosmetic applications, while contributing to a sustainable biotransformation approach.

- **Assisted Physical Methods**

Physical methods, such as ultrasound-, microwave-, or high hydrostatic pressure-assisted hydrolysis, are generally used as complementary strategies to enzymatic or chemical hydrolysis in order to improve process performance. Ultrasound, for instance, induces cavitation that disrupts protein structures and exposes more cleavage sites to enzymes. Microwaves accelerate denaturation and facilitate access to peptide bonds, whereas high hydrostatic pressure alters protein conformation and enhances reaction

kinetics (Jambrak *et al*., 2014). These innovative approaches help reduce hydrolysis time and limit the use of chemical agents, while increasing both the yield and quality of the hydrolysates

2.2.Physicochemical Characterization of Protein Hydrolysates

The physicochemical characterization of protein hydrolysates aims to determine their structural, compositional, and functional properties in order to evaluate their quality and potential applications. Among these, solubility, emulsifying, and foaming properties are particularly important, as they largely define the functionality and end-use of bioactive peptides in food, nutraceutical, and biotechnological applications.

- **Solubility**

  Solubility is one of the most critical functional properties of protein hydrolysates, as it directly affects other properties such as emulsification and foaming (Souissi *et al*., 2007). The enhanced solubility of hydrolysates is attributed to their smaller molecular size compared to intact proteins, as well as to the newly exposed ionizable amino and carboxyl groups generated during hydrolysis (Wilding *et al*., 1984). Consequently, solubility generally increases with a higher degree of hydrolysis (Quaglia *et al*., 1987b). The high solubility of fish protein hydrolysates is particularly valuable for applications such as the production of milk replacers for animals and the manufacture of peptones for microbial growth media (Hale & Bauersfeld, 1978).

- **Emulsifying Property**

  Proteins are amphiphilic molecules that have long been used in the food industry as emulsifiers. Protein hydrolysates enhance the formation and stabilization of oil-in-water emulsions because they contain both hydrophilic and hydrophobic functional groups (Fennema, 1996). The emulsifying capacity and stability of these hydrolysates largely depend on enzymatic specificity and the degree of hydrolysis (Paraman *et al*., 2007).

- **Foaming Property**

  The ability of proteins to form stable foams is also of major importance in the development of a wide variety of food products. The foaming capacity of proteins increases as surface tension decreases. These properties are mainly influenced by

protein type, the nature of solutes, and molecular interactions, including hydrophobic interactions, electrostatic forces, hydrogen bonding, and disulfide bridges.

2.6.Preparation of Fish Protein Hydrolysates

Fish by-products (skin, viscera, heads, muscles, and bones) were chopped and homogenized using a Retsch GM 200 grinder. The minced material was mixed (1:1, v/v) with 0.5 M Glycine-NaOH buffer (pH 10.0) and autoclaved at 120 °C for 15 min. Enzymatic hydrolysis was performed by adding 2% (v/v) crude enzymatic extract from *Bacillus mojavensis* FP2 (470.59 U/mL) at 50 °C under continuous agitation (120 rpm) for 2 h.

Aliquots were collected every 15 min, heated at 90 °C for 20 min to inactivate the enzyme, and centrifuged at 4500 rpm for 30 min to separate the soluble fraction (FPH), insoluble residue, and lipids (Figure 2). Hydrolysates were frozen at −20 °C overnight, freeze-dried for 21 h using a CHRIST Beta 2-8 LSCplus lyophilizer, and stored at 4 °C until use.

A non-hydrolyzed protein sample, obtained by stopping the reaction immediately after enzyme addition, was used as a control (FT).

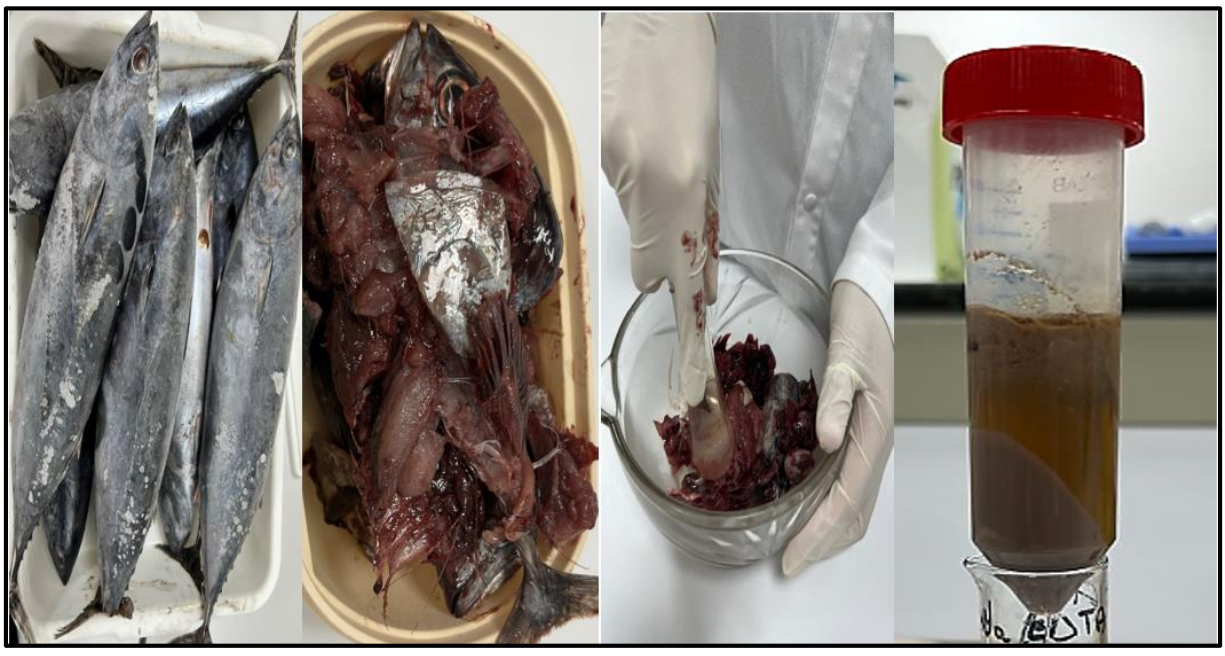


Figure 02 : Steps of fish protein hydrolysate production

2.7.Determination of Degree of Hydrolysis

The degree of hydrolysis (DH) was determined using the ninhydrin method to quantify free α-NH_2 groups. Briefly, 500 µL of hydrolysate solution (0.1 mg/mL) was mixed with 1 mL of ninhydrin solution. The mixture was heated at 84 °C for 5 min and then cooled on ice. Absorbance was measured at 507 nm.

A calibration curve was prepared using tyrosine solutions ranging from 0.05 to 3 mmol/mL (Figure 3). The DH was calculated according to the following equation :

$$\mathbf{DH\ (\%) = h / htot \times 100}$$

where *h* represents the number of cleaved peptide bonds (meqv/g protein) and *htot* is the total number of peptide bonds (meqv/g protein).

2.8.Characterization of Protein Hydrolysates

2.8.1. Solubility

The solubility of protein hydrolysates was determined over a pH range of 4.0 to 10.0. Hydrolysates (1%, w/v) were dissolved in distilled water, and pH was adjusted using 2 M NaOH and HCl (1:9, v/v). Solutions were stirred for 10 min at room temperature and centrifuged at 8000 g for 10 min. Nitrogen content was quantified using the Bradford method (1976). Solubility was calculated as follows:

Nitrogen solubility (%)=

(Nitrogen concentration in sample / Nitrogen concentration in supernatant) ×100

2.8.2. Emulsifying Property

Olive oil (5 mL) was mixed with 5 mL of protein hydrolysate solution (5%, w/v) and homogenized at 25,000 rpm for 2 min at room temperature. The emulsion was centrifuged at 2400 g for 3 min. The volumes of oil, emulsion, and aqueous fractions were measured, and emulsifying capacity was expressed as the volume of the emulsion fraction (mL) per gram of hydrolysate.

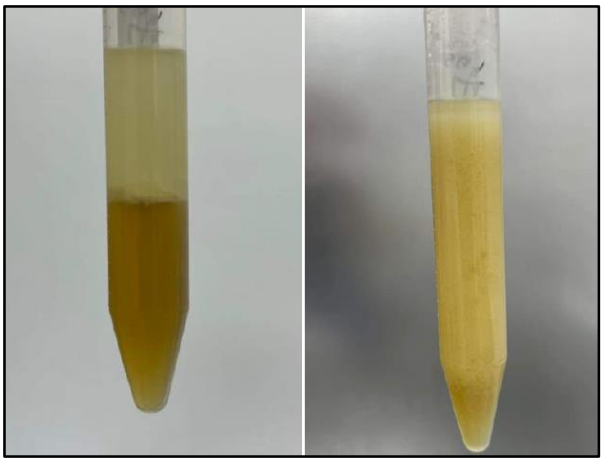


Figure 03 : Appearance of the emulsion after centrifugation

2.8.3. Foaming Property

A total of 0.1 g of each hydrolysate was dispersed in 10 mL of distilled water. The pH of the mixture was adjusted to 4.0, 6.0, 8.0, or 10.0 using 2 M NaOH and HCl (1:9, v/v). Samples were homogenized at 25,000 rpm for 5 min at room temperature using a homogenizer. Foam volume was recorded, and the foaming capacity of hydrolysates was calculated as follows:

Foaming capacity (%) = (Foam volume Initial / sample volume) ×100

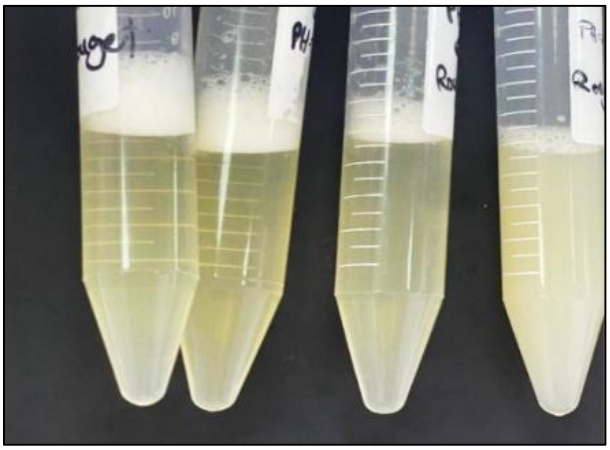


Figure 04 : Foaming capacity

- **Evaluation of Chapter Two :**

1. How can the term co-product be distinguished from the term waste ?

2. What are the main applications of fish protein hydrolysates in the food industry ?

3. What is the main advantage of using enzymes for the hydrolysis of fish proteins compared to chemical methods ?

- **The answer key**

1. A co-product is a valuable material intentionally generated during production, while waste is an unwanted residue with no intended value. Co-products can be reused or sold, whereas waste usually requires disposal.

2. Fish protein hydrolysates are mainly used as flavor enhancers, nutritional ingredients, and functional additives in food products. They improve taste, digestibility, and bioactive properties in soups, sauces, snacks, and dietary supplements.

3. The main advantage of enzymatic hydrolysis is its high specificity and mild reaction conditions, which preserve nutritional quality. Unlike chemical methods, enzymes avoid harsh chemicals and produce more controlled, high-quality hydrolysates.

3. Workshop 3 : Valorization of Crustacean Waste

The aim of this workshop is to formulate, modify, and valorize materials derived from biowaste, particularly crustacean shells, for their use as bio-adsorbents of organic and inorganic pollutants through adsorption processes. Furthermore, the study seeks to enhance their retention performance by applying both chemical and physical modifications

3.1. Composition of Chitin in Crustacean Shells

The composition of crustacean biowaste is directly influenced by factors such as species, sex, environmental growth conditions, and diet. Additionally, part of the observed variability arises from the analytical methods employed for determining the composition of biowaste. For a long time, crustacean residues from the agri-food industry were discarded into the sea after shell removal. Currently, the global annual production of chitin/chitosan is estimated at 10^9–10^{10} tons, which remains significantly lower than that of cellulose.

Chitin is a naturally occurring crystalline fiber belonging to the family of nitrogenous polysaccharides. Its chemical structure is very similar to that of cellulose, with the only difference being the presence of an acetamide group on the C2 position in chitin, instead of the hydroxyl group found in cellulose (Figure 1). Chitin is a high molecular weight linear polymer consisting of repeating units of D-glucosamine (GlcN) and N-acetyl-D-glucosamine (GlcNAc), linked through β-(1→4) glycosidic bonds. It is a nitrogenous, white, flexible material, permeable to water and air, biodegradable, and biocompatible. Due to its hydrophobic nature, chitin is insoluble in water and in most organic solvents. Generally, its degree of acetylation is approximately 90% or higher.

Chitosan, in contrast, is a cationic semi-crystalline polysaccharide that is rarely found naturally in marine sources. It is mainly obtained through the deacetylation of chitin. Chitosan consists of linear chains of repeating units of 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose, linked by β-(1→4) glycosidic bonds, with a degree of deacetylation greater than 50%. It is a natural material with a flaky, white, odorless appearance, of high molecular weight, non-toxic, renewable, biodegradable, and biocompatible. Furthermore, it exhibits significant chemical and biological reactivity, as well as diverse functional properties, including chelation ability, film-forming capacity, and strong adsorption potential.

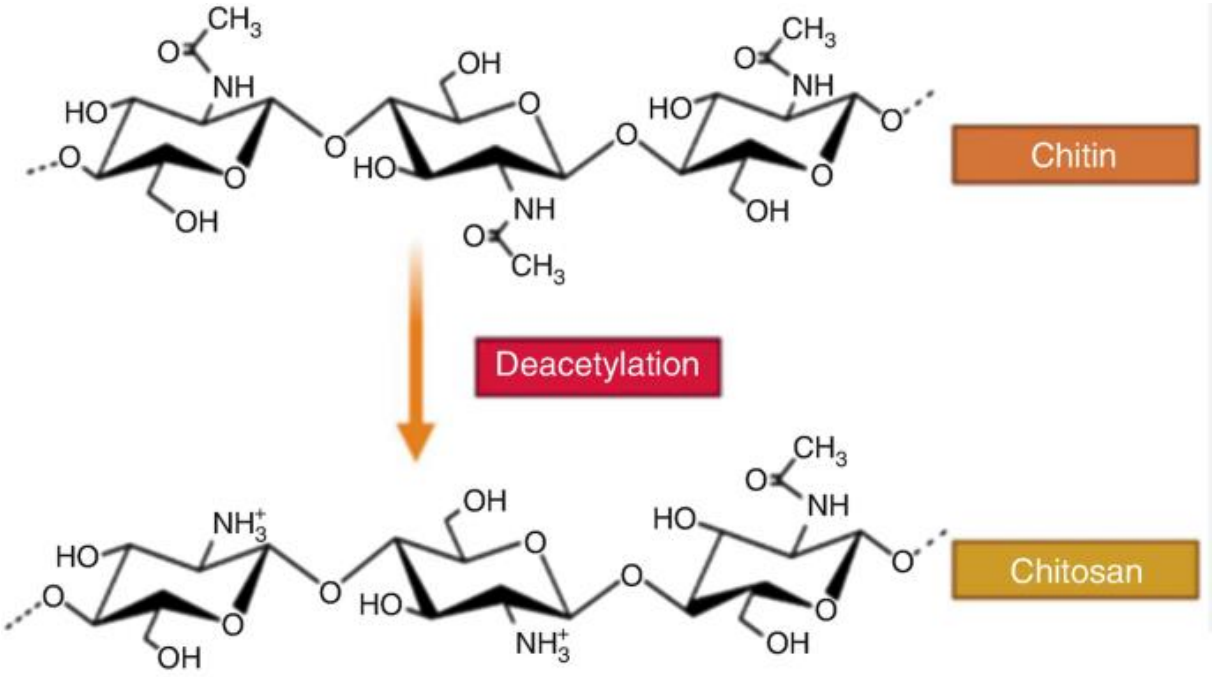


Figure 05 : Chitin and Chitosan

3.2.Extraction of Chitin

Chitin is found in crustaceans in the form of chitin–protein–mineral complexes, and several extraction protocols from exoskeletons have been proposed. In general, chitosan extraction is carried out using an acid–alkaline chemical process, which aims to remove proteins, minerals, and pigments in three main steps.

Prior to extraction, a pretreatment step is performed consisting of washing, drying, and grinding of the shells. The obtained material is then stored for further use. The main stages of chitin extraction include deproteinization and demineralization**,** with an optional step of decolorization (bleaching)**.** To obtain chitosan, an additional deacetylation step is required.

- **Deproteinization**

This step consists in removing 30–40% of proteins bound to chitin in the form of chitino-proteins through covalent bonds. Deproteinization can be carried out either by a harsh chemical method (alkaline treatment) or by a milder biological method (using proteolytic enzymes or fermentation).

- **Demineralization**

This step involves the removal of 30–50% of calcium carbonate present in crustacean shells through acid treatment under agitation. It is usually performed at room temperature, with reaction times ranging from 1 to 48 hours, depending on the type and concentration of acid used.

- **Decolorization (Bleaching)**

The presence of pigments, such as carotenoids, gives crustacean shells their characteristic pink-orange color. Decolorization is an optional step aimed at minimizing strong interactions between chitin and pigments, as well as eliminating residual pigments. This is typically achieved using oxidizing or bleaching agents.

3.3.Extraction of Chitosan

- **Deacetylation**

The deacetylation of chitin is carried out by hydrolysis under strongly alkaline conditions, most often using concentrated NaOH or KOH solutions (40–50%) at elevated temperatures. The yield of alkaline deacetylation depends on the efficiency of the previous steps, as well as on factors such as the amount, density, and particle size of the chitin. Operational parameters including temperature, reaction time, alkali concentration, and atmosphere (air or nitrogen) also significantly influence the process.

Figure 06 : Comparison of Colloidal Chitin, Demineralized Chitin, Deproteinized Chitin, and Chitosan from Shrimp Shells

3.3.1. Chitosan Formulation

The solubility of chitosan in dilute acids allows its exploitation in a wide range of forms. It is recognized as a versatile material that can be processed into various physical structures, including films, beads, biocomposites, hydrogels, and nanoparticles**.**

3.3.1.1.Film Preparation

Chitosan exhibits excellent film-forming properties, which can be achieved through different approaches, such as :

- Direct pervaporation of chitosan solvent,
- Cross-linking of chitosan with bifunctional reagents,
- Air-drying of chitosan solutions.

The ability of chitosan to form inter- and intramolecular hydrogen bonds as well as its glycosidic linkages enhances its filmogenic performance. Several protocols have been developed and optimized for the preparation of chitosan-based films for applications in packaging, biomedicine, and water treatment.

The biofilm preparation was carried out through the following steps:
i) Stirring 1 g of CS-II in 100 mL of 0.17 M acetic acid for 3 h at room temperature.
ii) Casting the resulting CS solution into Petri dishes (90/15 mm).
iii) Immersing the obtained CS film in 0.1 M NaOH for 1 h.
iv) Washing with distilled water until neutral pH was achieved.

Figure 07 : Photograph of the chitosan-based biofilm

3.3.1.2.Microcapsule Preparation

Chitosan beads can be synthesized using several techniques, mainly:

- Extrusion**,**
- Aerosol gelation**,**
- Emulsion methods**.**

These beads are widely applied in drug delivery, enzyme immobilization, and pollutant adsorption due to their high surface area and biocompatibility.

CS-II beads were prepared by dissolving 3 g of chitosan in 100 mL of 2% acetic acid under continuous stirring. The resulting viscous solution was then added dropwise into 500 mL of 0.5 M NaOH solution under moderate and continuous stirring. The formed beads were left to stand in the alkaline solution overnight. CS-II beads were subsequently filtered and thoroughly washed with distilled water until neutral pH was achieved. Finally, the beads were air-dried.

3.4.Chitooligosaccharides (CHOS)

Chitooligosaccharides (CHOS) are low-molecular-weight oligomers derived from chitosan, obtained either chemically or enzymatically. Chitosan can be hydrolyzed by:

- Acid hydrolysis**,**
- Enzymatic hydrolysis using glycosyl hydrolases (e.g., chitinases, chitosanases).

The degree of acetylation (FA)**,** molecular weight (MW)**,** polydispersity (PD**)**, and pattern of acetylation (PA) of the CHOS mixture depend on the source of chitosan and the specificity of the enzyme employed. By optimizing the enzyme–substrate combination.

Table 05 : Pictograms and indications of danger

Products	Hazard pictograms	Warnings
Sulfuric acid		Hazard to health Toxic Corrosion
Hydrochloric acid		Corosive harmful and irritating
Sodium hydroxid		Corosive
Hydrogen peroxide		Corosive harmful and irritating

- **Evaluation of Chapter three :**

1. What is chitin and where is it commonly found in aquatic bioresources ?
2. Mention two main applications of chitosan in the food or pharmaceutical industry.
3. Why are chitooligosaccharides considered bioactive compounds ?
4. Give two environmental benefits of valorizing crustacean shells instead of discarding them.

- **The answer key**

1. Chitin is a natural polysaccharide that forms the structural component of many aquatic organisms. It is mainly found in the shells of crustaceans such as shrimp, crab, and lobster, as well as in squid pens.

2. In the food industry, chitosan is used as an antimicrobial and antioxidant coating to preserve freshness. In the pharmaceutical field, it is applied for drug delivery and wound healing due to its biocompatibility.

3. Chitooligosaccharides are considered bioactive because they show antioxidant, antimicrobial, and anti-inflammatory properties. Their small size allows them to be easily absorbed and act effectively in biological systems.

4. Valorizing crustacean shells reduces organic waste pollution and decreases landfill usage. It also promotes resource recovery by transforming by-products into valuable biomaterials.

4. Workshop 4 : Functional Activities of Fish Protein Hydrolysates

4.1.Biological Activities of Protein Hydrolysates

Many peptides produced *in vitro* through the hydrolysis of animal or plant proteins exhibit interesting biological activities that can modulate several regulatory functions in the body. Numerous studies have reported the bioactivities of peptides, including antioxidant, hypoglycemic, and antimicrobial properties.

The investigation of the functional activities of fish protein hydrolysates can serve several objectives :

- **Evaluation of biological efficacy** : Understanding how fish hydrolysates interact with the human or animal body, including studies on absorption, bioavailability, and the physiological effects of bioactive compounds present in the hydrolysates.
- **Identification of bioactive compounds** : Identifying and characterizing peptides, amino acids, lipids, vitamins, minerals, and other bioactive molecules in fish hydrolysates, as well as assessing their health-related effects.
- **Functional properties** : Assessing the functional properties of fish hydrolysates, such as antioxidant, anti-inflammatory, antimicrobial, hypocholesterolemic, and immunomodulatory activities.
- **Food and medicinal applications** : Exploring potential applications of fish hydrolysates in functional foods, dietary supplements, medicine, and nutraceuticals.
- **Process improvement** : Optimizing hydrolysate production methods to maximize functional activity and biological efficiency.
- **Food safety** : Evaluating the safety of fish hydrolysates in terms of toxicity and potential adverse effects.

In summary, studying the functional activities of fish protein hydrolysates aims to provide a better understanding of their health effects in humans and animals, as well as their potential as functional ingredients across diverse sectors, ranging from food to medicine.

4.1.1. DPPH free radical scavenging activity

The antioxidant activity of the different protein hydrolysates was determined according to the method described by Bersuder *et al*. (1998). A volume of 500 µL of hydrolysate solution at different concentrations (2, 5, and 10 mg/mL) was mixed with 375 µL of 96% ethanol and 125 µL of a 0.8 mM DPPH solution prepared in ethanol. The mixture was incubated for 1 h at room temperature. The absorbance was measured at 517 nm using a spectrophotometer. The percentage of DPPH inhibition was calculated according to the following equation :

$$\% \text{ DPPH inhibition} = (Absc + Absb - AbsE/Absc) \times 100$$

where Absc is the absorbance of the control (distilled water instead of hydrolysate), Absb is the absorbance of the blank (ethanol and hydrolysate without DPPH), and *AbsE* is the absorbance of the test sample.

4.1.2. Antibacterial activity

The pathogenic strains used for the antibacterial activity assay were *Bacillus subtilis* ATCC 3289, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 5362, and *Escherichia coli* ATCC 25922. These strains were kindly provided by Dr. Meriem Meliani from the Laboratory of Aquaculture and Bioremediation, University of Oran 1. All strains were preserved at –20 °C in 50% glycerol.

The agar well diffusion method was employed to assess the antibacterial activity of FPH and CFPH protein hydrolysates. Petri dishes containing Mueller–Hinton agar were inoculated by spreading fresh bacterial suspensions (~10^7 cells/mL) over the agar surface using sterile swabs. Wells of 5 mm in diameter were then punched into the agar, and 50 µL of hydrolysate solution (100 mg/mL) was introduced into each well. After incubation at 37 °C for 24 h, the diameters of the inhibition zones were measured, and antibacterial activity was expressed as the ratio of inhibition zone diameter to well diameter.

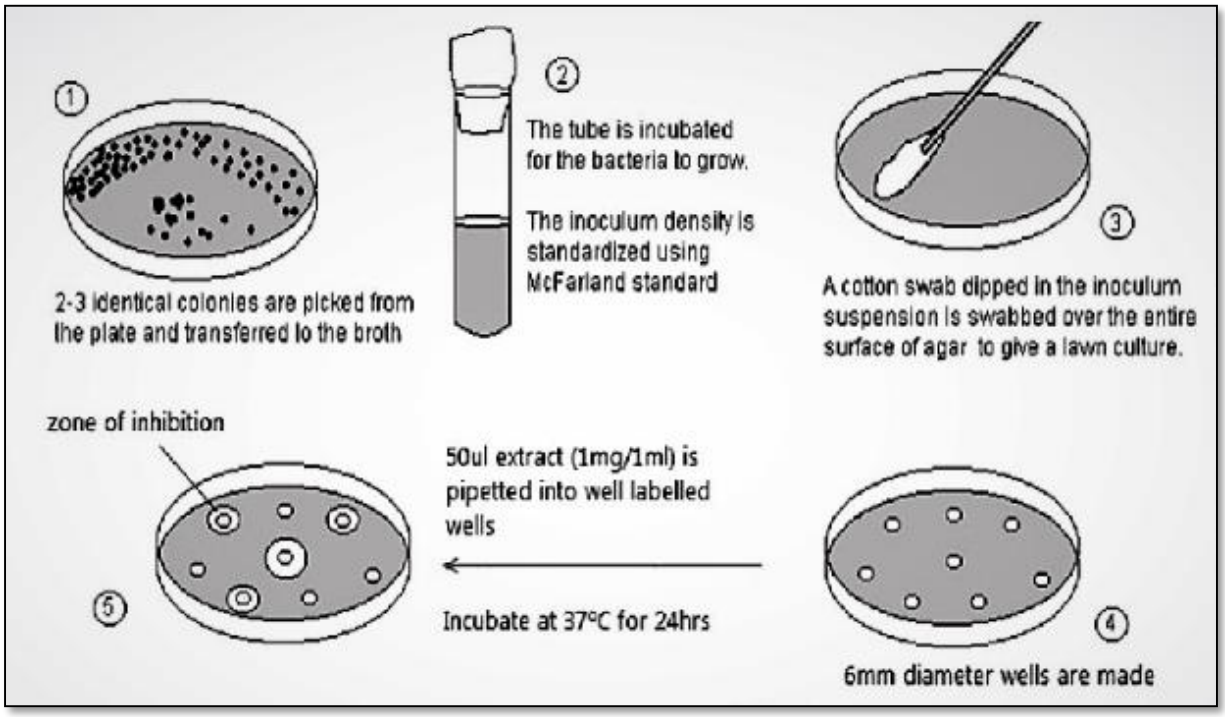


Figure 08 : Demonstration of antimicrobial activity using the well diffusion method

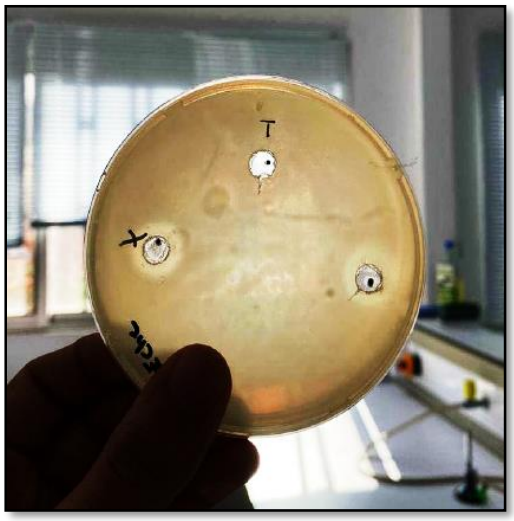


Figure 09 : Demonstration of antimicrobial activity using the well diffusion method

- **Evaluation of Chapter four :**

1. Why do fish protein hydrolysates show antioxidant activity ?
2. Mention two mechanisms by which bioactive peptides can inhibit bacterial growth.

- **The answer key**

1. Fish protein hydrolysates exhibit antioxidant activity because their peptides contain amino acids capable of donating electrons or hydrogen atoms to neutralize free radicals. They can also chelate metal ions that catalyze oxidation reactions, thereby reducing oxidative stress in biological systems.
2. Bioactive peptides can inhibit bacterial growth by integrating into and disrupting the bacterial cell membrane, causing leakage of cellular contents. They can also interfere with key intracellular processes, such as enzyme function or nucleic-acid synthesis, ultimately preventing the bacteria from surviving or multiplying.

5. Evaluation, Discussion and Conclusion

The assessment of this workshop will be carried out according to the following criteria :

- **Final Report (30 %)** : At the end of the workshop sessions, students will submit a written report. This document should include a summary and introduction, a description of the materials used, and a detailed account of the experimental methods. Students are required to present the obtained results, analyze and discuss them with reference to relevant scientific literature, and conclude with a list of bibliographic references.
- **Laboratory Notebook (20 %)** : Students must maintain a detailed record of all activities performed during the sessions. This includes the materials employed, the procedures followed, and any modifications introduced. The notebook should also document the results obtained and any difficulties encountered. Furthermore, students are encouraged to provide constructive feedback, identifying the strengths and weaknesses of the workshop to support future improvements.
- **Attendance and Participation (10 %)** : Evaluation will take into account student presence, proper handling of equipment, seriousness, and active participation during the sessions.
- **Quizzes (40%)** : Students will complete quizzes throughout the workshop to assess their understanding and level of comprehension.

Bibliographic reference

- **Anson, M. L. (1938).** The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *Journal of General Physiology*, 22(1), 79–89.
- **Arumugam, N., Almansour, A. I., Suresh, K. R., Altaf, M., Padmanaban, R., Sureshbabu, P., Angamuthu, G., Kotresha, D., Manohar, T. S., & Venkatesh, S. (2018).** Spiropyrrolidine/spiroindolizino[6,7-b]indole heterocyclic hybrids: Stereoselective synthesis, cholinesterase inhibitory activity and molecular docking study. *Bioorganic Chemistry*, 79, 64–71.
- **Berg, J. M., Tymoczko, J. L., Gatto, G. J., & Stryer, L. (2019).** Biochemistry. *Biochemistry*, 9, 1–1200.
- **Bersuder, P., Hole, M., & Smith, G. (1998).** Antioxidants from a heated histidine–glucose model system. I : Investigation of the antioxidant role of histidine and isolation of antioxidants by high-performance liquid chromatography. *Journal of the American Oil Chemists' Society*, 75(2), 181–187.
- **Bisswanger, H. (2017).** Enzyme kinetics : Principles and methods. *Enzyme Kinetics*, 3, 1–350.
- **Bradford, M. M. (1976).** A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2), 248–254.
- **Caruso, G., Floris, R., Serangeli, C., & Di Paola, L. (2020).** Fishery wastes as a yet undiscovered treasure from the sea : Biomolecules sources, extraction methods and valorization. *Marine Drugs*, 18(12), 622.
- **Chalamaiah, M., Hemalatha, R., & Jyothirmayi, T. (2012).** Production, purification and functional properties of fish protein hydrolysates : A review. *Food Chemistry*, 135(4), 3020–3038.
- **Doudna, J. A., & Cech, T. R. (2002).** The chemical repertoire of natural ribozymes. *Nature*, 418(6894), 222–228.
- **FAO. (2024).** The state of world fisheries and aquaculture 2024. *FAO Fisheries and Aquaculture Report*, 2024, 1–260.
- **Fennema, O. R. (1996).** Food chemistry. *Food Chemistry*, 3, 1–1067.
- **Hale, M. B., & Bauersfeld, P. J. (1978).** Preparation of a menhaden hydrolysate for possible use in a milk replacer. *Marine Fisheries Review*, 40(8), 14–18.

- **Korhonen, H., & Pihlanto, A. (2006).** Bioactive peptides : Production and functionality. *International Dairy Journal*, 16(9), 945–960.
- **Kristinsson, H. G., & Rasco, B. A. (2000).** Fish protein hydrolysates : Production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition*, 40(1), 43–81.
- **Narayana, K. J. P., & Vijayalakshmi, M. (2009)**.
- **Nelson, D. L., & Cox, M. M. (2021).** Lehninger principles of biochemistry. *Biochemistry*, 8, 1–1320.
- **Paraman, I., Hettiarachchy, N. S., Schaefer, C., & Beck, M. I. (2007).** Hydrophobicity, solubility, and emulsifying properties of enzyme-modified rice endosperm protein. *Cereal Chemistry*, 84(4), 343–349.
- Petrova, I., Tolstorebrov, I., & Eikevik, T. M. (2018). Production of fish protein hydrolysates step by step : Technological aspects, equipment used, major energy costs and methods of their minimizing. *International Aquatic Research*, 10(3), 223–241.
- **Quaglia, G., & Orban, E. (1987).** Influence of the degree of hydrolysis on the solubility of protein hydrolysates from sardine (*Sardina pilchardus*). *Journal of the Science of Food and Agriculture*, 38(3), 271–276.
- **Rai, A. K., Sanjukta, S., Chourasia, R., Bhat, I., Bhardwaj, P. K., & Sahoo, D. (2017).** Production of bioactive hydrolysate using protease, β-glucosidase and α-amylase of *Bacillus* spp. isolated from kinema. *Bioresource Technology*, 235, 358–365.
- **Souissi, N., Bougatef, A., Triki-Ellouz, Y., & Nasri, M. (2007).** Biochemical and functional properties of sardinella (*Sardinella aurita*) by-product hydrolysates. *Food Technology and Biotechnology*, 45(2), 187–194.
- **Theysgeur, S., Cudennec, B., Deracinois, B., Perrin, C., Guiller, I., Lepoudère, A., Flahaut, C., & Ravallec, R. (2021).** New bioactive peptides identified from a tilapia by-product hydrolysate exerting effects on DPP-IV activity and intestinal hormones regulation after canine gastrointestinal simulated digestion. *Molecules*, 26(1), 136.
- **Voet, D., Voet, J. G., & Pratt, C. W. (2016).** Fundamentals of biochemistry : Life at the molecular level. *Biochemistry*, 4, 1–1200.
- **Wilding, P., Lillford, P. J., & Regenstein, J. M. (1984).** Functional properties of proteins in foods. *Journal of Chemical Technology and Biotechnology*, 34(3), 182–189.

- **Zamora-Sillero, J., Gharsallaoui, A., & Prentice, C. (2018).** Peptides from fish by-product protein hydrolysates and its functional properties: An overview. *Marine Biotechnology*, 20(2), 118–130.
- **Zhao, Y., Zhang, Y., Wang, G., Han, R., & Xie, X. (2016).** Effects of chlorpyrifos on the gut microbiome and urine metabolome in mouse (*Mus musculus*). *Chemosphere*, 153, 287–293.

- ## Appendix

In the preparation of this educational material, the artificial intelligence tool *Perplexity* was utilized to translate the content into scientific English. *Perplexity* is an AI-driven conversational platform that combines an advanced search engine with a language agent capable of analyzing and synthesizing real-time information from the web. By leveraging its natural language processing capabilities, the tool facilitated the generation of accurate, coherent, and contextually relevant translations for scientific purposes, while preserving the original meaning and clarity of the text. This resource ensured a reliable translation process, supported by an intuitive interface and access to verifiable references.

- ## Glossary of terms

- **GenBank**

  GenBank is a DNA sequence database that contains all publicly available nucleotide sequences and their corresponding protein translations.

- **Luria-Bertani (LB) medium**

  It was first developed by Bertani, who originally named it *lysogeny broth* in his initial publication.

- **Bioactive peptides**

  Bioactive peptides are short sequences of amino acids that exert beneficial biological effects.

- **Substrate**

  A substrate is a substance that undergoes a chemical or biological transformation as a result of the action of an enzyme, chemical reagents, or microorganisms.

- **Hydrophilic and Hydrophobic**

  The terms *hydrophilic* and *hydrophobic* are used to describe the interactions of a substance with water.

- **Bioactive Peptides**

  Bioactive peptides are short chains of amino acids that exert beneficial biological effects on the organism when consumed. These peptides are often derived from dietary proteins but can also be synthesized in the laboratory.

- **Buffer Solutions**

  Buffer solutions are typically prepared by dissolving a weak acid and its salt (or a weak base and its conjugate salt) in deionized water, followed by adjustment of the pH to the

desired value using a strong acid or a strong base, if necessary. Commonly used buffers include phosphate buffer, Tris-HCl buffer, cacodylate buffer, and HEPES buffer.

Annex :

Buffers and Reagents

- **50 mM Potassium Phosphate Buffer, pH 7** : Prepared by mixing 37.7 ml of 50 mM dipotassium hydrogen phosphate (K_2HPO_4) solution with 62.3 ml of 50 mM potassium dihydrogen phosphate (KH_2PO_4) solution.
- **50 mM Glycine–NaOH Buffer, pH 9.0–12.0** : 75 mg of glycine were dissolved in distilled water, and the pH was adjusted with NaOH (2 M). The final volume was adjusted to 20 ml.
- **50 mM Tris-HCl Buffer, pH 8** : 121 mg of Tris were dissolved in distilled water, and the pH was adjusted with HCl (1:9 v/v). The final volume was adjusted to 20 ml.
- **Bradford Reagent** : Prepared by mixing 5 mg of Coomassie Brilliant Blue G-250, 2.5 ml of 95% ethanol, and 5 ml of 85% phosphoric acid. The final volume was adjusted to 50 ml with distilled water, and the solution was filtered.
- **Ninhydrin Solution** : Composed of 0.8 g of ninhydrin, 80 ml of ethanol, 10 ml of glacial acetic acid, and 2 g of cadmium chloride.

.