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Optimization of yeast protease production and characterization of the enzyme

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Dedication and Acknowledgments

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Dedication

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Dedication

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Abbreviations

SSF: Solid State Fermentation

TP: Tomato Pomace

BW: Bread Waste

YPGA: Yeast extract-Peptone-Gelose-Agar

pH: potentiel d'hydrogène

TCA: TriChloroacetic Acide

PBD: Plackette-Burman's Design

UI : Unité Internationale

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Introduction

In industry, enzymes play a crucial role due to their specificity, sensitivity, and eco-friendly nature, thereby significantly contributing to the economy. Industrial enzymes of microbial origin are used in various sectors such as food, pharmaceuticals, textiles, etc. (Swamy, 2022; Molina-Espeja et *al*., 2023; Sharma & Vimal, 2023). Their use leads to reductions in energy consumption, waste generation, and environmental pollution, aligning with eco-friendly practices and strict regulations, thereby boosting the growth of the global market(Guerrand, 2018; Ramesh et *al*., 2020).

In Algeria, enzymes essential to the industry are often imported and expensive, whereas they could be produced locally by microorganisms such as yeasts. It would therefore be interesting to develop the production of enzymes from local strains using cost-effective processes.

Commercial enzymes are historically from Molds and bacteria sources (Sharma & Vimal, 2023; Swamy, 2022), Among the enzymes, proteases are considered as one of the most important groups of industrial enzymes, representing about 60% of the global market share (Alahmad Aljammas et *al*., 2022; Nadeem et al., 2020). Moreover, proteases may be classified into acidic, neutral and alkaline (basic) proteases according to the optimal pH (Zhu et *al*., 2019). Acid proteases are one of the most widely used categories of enzymes that is widely employed in food and beverage industries(Usman et *al*., 2021).

The role of aspartic proteases in biotechnology and medicine is important. Previous studies have concentrated on the enzymes from *Candida* species because of their importance as virulence factors, even though there is ample evidence that numerous *non-Saccharomyces* yeasts produce extracellular proteases. These days, their low pH activities attract more attention to their applications in industrial processes (Schlander et *al*., 2017). The characteristics of novel acid proteases produced by yeasts isolated from agricultural food waste are presented here. These yeasts are identified as *Clavispora lusitaniae* and *Rhodotorula mucilaginosa*.

This is, as far as we have knowledge, the first detailed description of that enzyme kind produced from these two strains.

Currently, to produce proteases, solid-state fermentation (SSF) or submerged fermentation (SMF) is used (Contesini et *al*., 2018; Dos Santos Aguilar & Sato, 2018; Razzaq et *al*., 2019). However, several characteristics make solid-state fermentation more preferred than submerged

fermentation, including cost-effectiveness, simplicity, higher yield, product stability, lower sterility requirements, etc.,(Contesini et *al*., 2018).

Today, solid-phase processes are increasingly attractive worldwide, especially for the valorization of agricultural by-products for the production of various products and substances (Van Dyk et *al*., 2013), such as tomato waste and leftover bread.

For the industrial use of enzymes, it would be beneficial to enhance their production and stability through optimization methods based on statistical designs (Bennamoun et *al*., 2004), such as Plackett-Burman designs (1946), Box-Wilson designs (1951), Box-Behnken designs, and response surface methodology (Amenaghawon et *al*., 2013; Benkahoul et *al*., 2020; Bennamoun et *al*., 2004; Prajapati et *al*., 2015).

The main objectives of our work are:

- ✓ Protease production using the yeasts *Clavispora lusitaniae* and *Rhodotorula mucilaginosa* cultivated in solid-state fermentation (SSF) with leftover bread and tomato waste. These by-products are rich in carbohydrates and proteins, promoting good yeast growth and enzyme production.
- ✓ Optimize of protease production by applying the statistical designs of Plackett and Burman (1946).
- ✓ Characterize the protease from *Clavispora lusitaniae* and *Rhodotorula mucilaginosa*.

Our work consists of two parts. The first part is a literature review divided into three chapters: the first details the current state of knowledge on yeasts, and the second focuses on the study of proteases and their substrates. The second part addresses the methodology used in this study, and the discussion of the results. Finally, we conclude with general summary and future perspectives.

Literature review

Chapter one

Yeasts

1 Yeast

1.1 Generalties

Yeasts are unicellular eukaryotic microorganisms belonging to the kingdom of microscopic fungi. They have almost the same organelles as a mature eukaryotic cell (animal cell), such as the nucleus, the Golgi apparatus, the mitochondria, and the endoplasmic reticulum. They reproduce mainly by budding and sometimes by fission (Monroy Salazar et *al*., 2016).

Yeasts can survive in aerobic or anaerobic environments and are therefore described as facultative anaerobes. However, they generally prefer aerobic conditions for propagation, because the production of carbon dioxide and energy from oxygen and sugars is more efficient under these conditions.

Yeasts are mesophilic organisms, capable of tolerating a wide range of temperatures. However, they generally do not grow at temperatures above 40°C, their optimal temperature is generally between 25 and 30°C. There are approximately 500 species grouped into 60 genera (Labrecque, 2003).

1.2 Advantages of yeast over other microorganisms

Yeasts are widely recognized for their safety and effectiveness in industrial fermentation, which makes them better known than other microorganisms. They are not affected by viruses (phages) and are easy to recover due to their large size. In addition, the genetic stability of yeasts ensures excellent fidelity of fermentation processes, and the in-depth understanding of their cellular physiology facilitates their use(Labrecque, 2003).

They have several advantages over bacterial cells for expressing human proteins: they grow faster, are easier to manipulate genetically, and being eukaryotes, they modify proteins in the same way as human cells. Additionally, yeast cells can be easily lysed to purify proteins (Site 1).

1.3 Morphology and Structure

Yeast has a very simple structure since it is a unicellular organism. Sometimes this single cell forms a pseudomycelium, where yeast cells appear in a chain. The shape of a yeast cell can be elliptical, round or spherical, measuring between 3 to 15 µm in length and between 2 to 8 µm in width. Yeast is larger than most bacteria. It is immobile, possessing neither flagella nor other organs of locomotion (Baniya, 2023).

The yeast cell has a cell wall and a nuclear membrane, but, unlike plant cells, it does not contain chloroplasts (Patterson et *al*., 2023). The cytoplasm contains various cellular organelles, such as the Golgi apparatus, ribosomes, endoplasmic reticulum, mitochondria, nucleus, and vacuole (Baniya, 2023). Cell morphology can be easily observed using a ×40 objective on a fresh preparation. Yeast colonies are usually white, although sometimes they can be pink or red (Guiraud, 2016).

1.4 Use of yeast by humans

Among the different microorganisms, yeast has proven to be the most useful microorganism for humans. Its fermentation properties have been exploited for years. Its uses range from the food industry to the petroleum industry. Some of its uses are: Protein production, Biofuel, Baking industry (Baniya, 2023; Guadalupe-Daqui et *al*., 2021). Nowadays, many species of yeast are used to produce significant quantities of enzymes, particularly proteases (Ogrydziak, 1993) **(table 1)**.

Table 1: protease-producing yeast strains.

Levarian strain	**Types of proteases**	**References**
Clavispora lusitaniae	Acid protease	(Djekrif et *al*., 2024)
Rhodotorula mucilaginosa L7	Acid protease	(Lario et *al*., 2015)
Candida albican	Acid protease	(Ogrydziak, 1993)

Yarrowia lipolytica	Alkaline protease	(Ogrydziak, 1993)
Candida humicola	Acid protease	(Lario et *al*., 2015)

1.5 Nutrient Growth Factors and Enzyme Production

Like all living things, yeasts need nutrients to survive, grow, and also to produce enzymes. They must find in their environment all the elements necessary for cellular synthesis as well as favorable physicochemical conditions (Guiraud, 2016).

- **Carbon sources:** glucose, sucrose, starch, fructose.

The carbon source is one of the important factors affecting enzyme production, particularly when it acts as an enzyme inducer (Chaud, 2016; Dakhmouche, 2016).

- **Nitrogen source:** peptone, yeast extract, glutamine, purine bases and pyrimidines.

Organic sources influence the growth and fermentation activity of yeasts (CHAOUR Housna, 2021).

- **Salts:** K H_2PO_4, K_2HPO_4, $MgSO_4$, $7H_20$ and $CaCl_2$, 6 H_2O.

Mineral salts are not only important for the growth of microorganisms, they are also important for the production of enzymes and their activity (Dakhmouche, 2016).

- **Trace elements:** Ca^{2+}, Mg^{2+}, Mn^{2+}**,** Zn^{2+}**.**

Ca^{2+} ions play important roles in the metabolism and physiology of microorganisms (Dakhmouche, 2016).

1.6 Solid fermentation and liquid fermentation

Yeasts have the capacity to develop in solid or liquid fermentation and produce numerous biomolecules, in particular proteases. However, solid-state fermentation (SSF) has many advantages over liquid-based fermentation (SMF). These advantages include: simplicity, low cost, higher enzyme yield, concentrated nature of the solid substrate, reduced contamination due to low

moisture content, easier purification, and better circulation of liquid. Oxygen and less effort in post-treatment as well as the possibility of using several agro-industrial wastes as a relatively inexpensive substrate. Additionally, SSF is particularly suitable for the production of fungal enzymes (Djekrif et *al*., 2024; López-Trujillo et *al*., 2023).

1.7 Production of microbial enzymes for industrial use

The production of microbial enzymes has many advantages for industries seeking to improve their processes, reduce their environmental impact and create new products. Currently, the use of yeasts for the production of yeasts offers a considerable economic advantage, since they can be cultivated by fermentation on various agricultural or industrial wastes such as sugar cane bagasse, grape waste, wheat straw or barley, pineapple waste, orange peel, tomato waste, and wheat bran. Additionally, yeast strains can be isolated locally. These enzymes provide a sustainable and environmentally friendly option, while having great economic and commercial value. For their production, inexpensive and ecological biotechnological processes have been developed, allowing the use of agro-food waste as a growing substrate (waste recovery) (Djekrif et *al*., 2024; López-Trujillo et *al*., 2023).

Chapter two

Proteases and substrate

2 Proteases

The largest family of proteolytic enzymes, proteases, also go by the names proteinases or peptidases. They are responsible for breaking down peptide bonds in proteins and polypeptides into smaller fragments. Owing to their diverse structural and functional makeup, these enzymes perform out an extensive range of functions, such as immune system cascade development, nutrition digestion, and intracellular protein synthesis or recycling (Dunming et *al*., 2019; Shankar et *al*., 2021). Their features vary, including substrate selectivity, active site and catalytic mechanism, pH and temperature optimums, and stability profile (Dunming et *al*., 2019)

2.1 Proteases Classification

The Enzyme Commission (CE) classifies proteases as hydrolases (group 3), which hydrolyze peptide bonds (subgroup 4) (Dunming et *al*., 2019). Proteases were first divided into two types: endopeptidases, which target internal peptide bonds, and exopeptidases (aminopeptidases and carboxypeptidases), whose activity is regulated by the NH2 and COOH termini of their corresponding substrates. However, the availability of structural and mechanistic data on these enzymes made new categorization systems available (López-Otín & Bond, 2008). Based on proteolytic mechanism, Proteases are classified into six different categories: serine, threonine, cysteine, aspartic, metalloprotease, and glutamic acid proteases, but glutamate proteases have not yet been found in mammals (Dunming et *al*., 2019; López-Otín & Bond, 2008). Alternatively, Proteases can be classed as acidic, neutral, or alkaline (basic) depending on their ideal pH. Acid proteases have pH optima ranging from 2.0 to 5.0, neutral proteases have pH optima around 7.0, and alkaline proteases have pH optima ranging from 8.0 to 11.0(Dunming et *al*., 2019)**(Figure1)**.

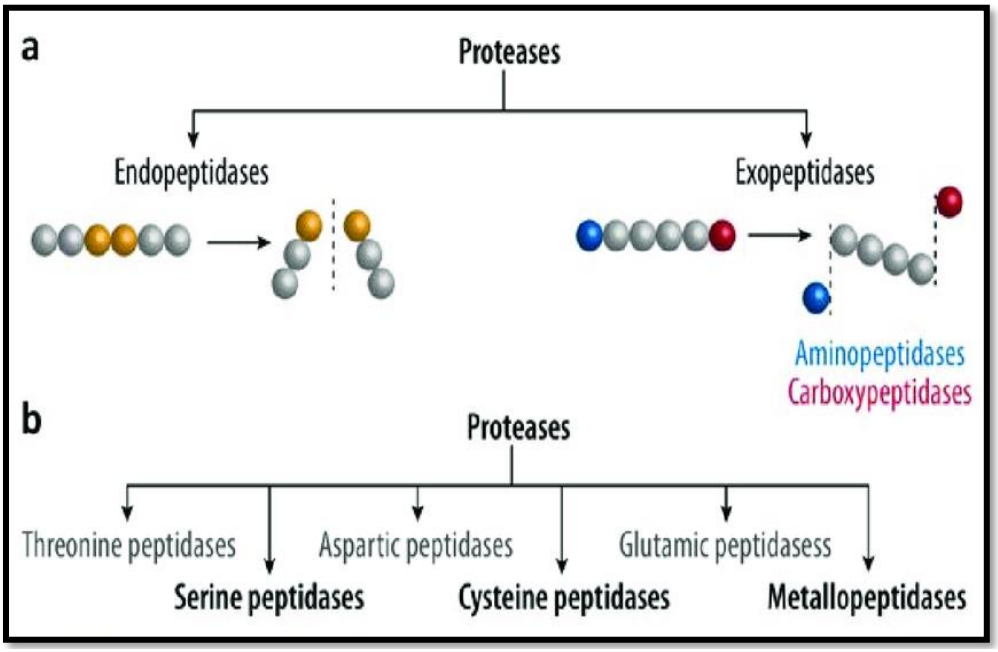

Figure 1: Classification of proteolytic enzymes (Ramírez-Larrota et *al*., 2022)

2.2 Mode of action-Structure and characteristics of proteases

Proteases are diverse and serve a variety of biological functions. Their structures are likewise highly diverse. However, they all, like enzymes in general, contain an active site that assures the hydrolysis activity of peptide bonds, as well as a substrate recognition site that only acts on the target protein (Site 2).

Human proteases are classified into five mechanistic types based on their hydrolytic catalytic mechanism: aspartate, cysteine, metallo, serine, and threonine proteases (Boon et *al*., 2020).

2.2.1 Mode of action

Generally, proteases use water at their active site in one of two ways:

1. **General acid-base catalysis:** Proteases activate water molecules, increasing their nucleophilicity and attacking cleavable peptide bonds.
2. **Covalent catalysis:** an activated protein side chains, such as serine and cysteine, function as nucleophiles and pass through the covalently bonded acyl enzyme intermediate. Water molecules then dissolve the intermediate, releasing the bound N-terminus substrate fragment.

Aspartate, glutamate, and metallopeptidases employ acid-base catalysis, while serine, cysteine, and threonine peptidases use covalent catalysis (Ramírez-Larrota et *al*., 2022).

2.2.2 Structure and Characteristics

2.2.2.1 Aspartic Proteases

Also known as Aspartic proteinases (EC 3.4.23), aspartyl proteinases or acid proteases, they are Endopeptidase with two aspartatic acid residues in the active site important for their catalytic activity (Mamo & Assefa, 2018) [(the conserved motif essential for catalytic activity of APs: Asp-Ser-Gly (DSG) or Asp-Thr-Gly (DTG) (Sadia Gull, 2024)].These enzymes catalyze their substrates by binding an active water molecule to aspartate via an H-bond interaction(Bhakat, 2021). Their molecular weight is 30-45 KDa, and their optimal temperature range is 40-55 KDa. Their peak activity level is 3-5 pH. Aspartic protease has an isoelectric point that ranges from 3 to 4.5. Pepstatin inhibits them (Mamo & Assefa, 2018; Shankar et *al*., 2021) **(Figure 02**).

The Acidic proteases are divided into three families: pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses and they have been placed in clan AA (Mamo & Assefa, 2018).

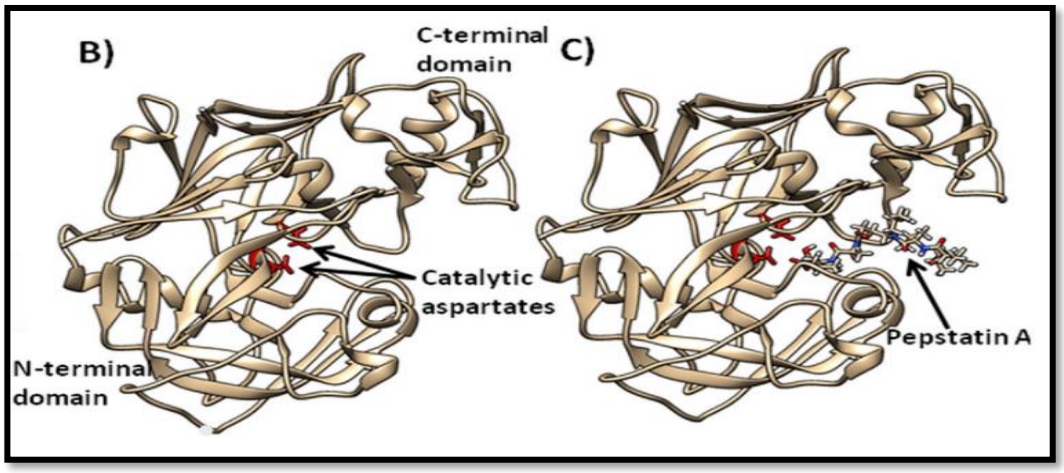

Figure 2: Structural model of aspartic protease. **B)** Structural model of aspartic protease, showing N-terminal and C-terminal domains and catalytic aspartates (marked in red colour), **C)** Structural model of pepstatin A bound aspartic protease (Kavya Purushothaman, 2021).

2.2.2.2 Glutamic Proteases

Glutamic peptidases, members of the MEROPS family G1, are a separate set of peptidases distinguished by a catalytic dyad composed of a glutamate and a glutamine residue, optimum activity at acidic pH, and resistance to the microbial-derived protease inhibitor pepstatin (Jensen et *al*., 2010).The glutamic peptidase family is assigned two clans, GA and GB, and comprises six subfamilies(Oda & Wlodawer, 2023) **(Figure 03)**.

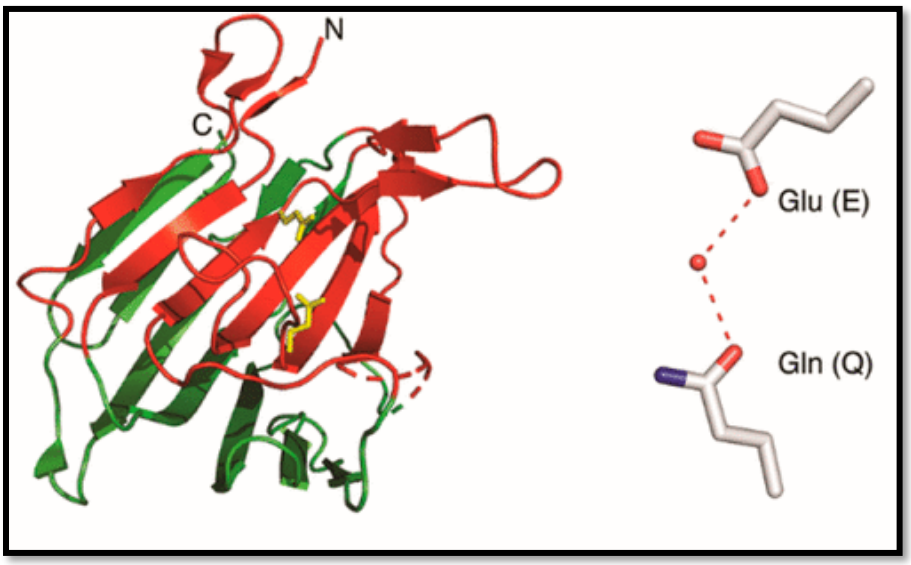

Figure 3: Structural model of glutamic protease (Oda & Wlodawer, 2023).

2.2.2.3 Metalloproteases

Metalloproteases (EC 3.4.24) are highly diversified types of proteases. They include enzymes from several sources, including collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Mamo & Assefa, 2018). They are a vast class of peptidases with an active site metal, commonly zinc (Ramírez-Larrota et *al*., 2022). Enzyme activity requires zinc atoms, and protein structure preservation requires calcium atoms **(Figure4)**.

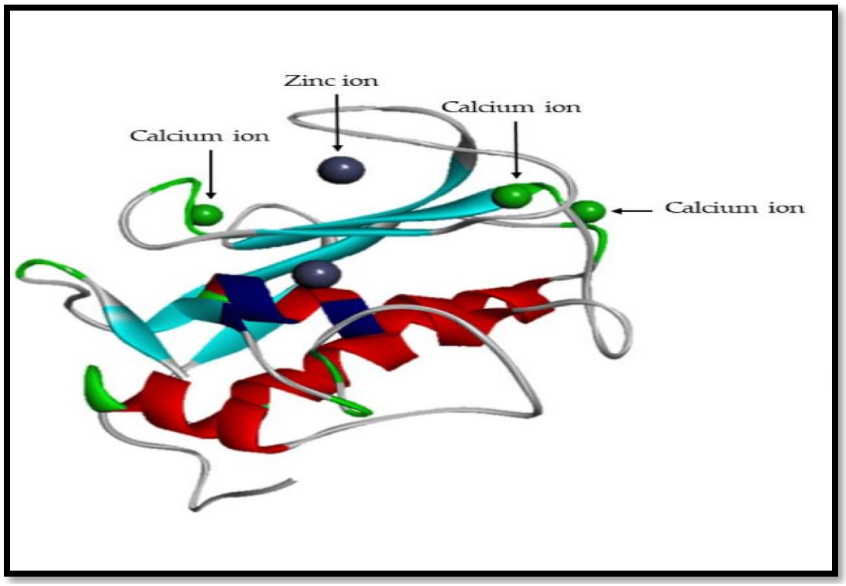

Figure 4: Structural model of metalloproteases (Laronha & Caldeira, 2020).

They have ideal pH ranges of pH 5.0–9.0 and are sensitive to metal-chelating reagents like EDTA (Ash & Mishra, 2023).The temperature optimum of Metalloproteases ranges between 5-85 and and molecular weight in the range of 19-27 KDa (Mamo & Assefa, 2018).

2.2.2.4 Serine Proteases

Serine proteases. Serine proteases (EC 3.4.21) are identified by possessing a serine group in their active site. They are often found in viruses, bacteria, and eukaryotes, indicating their importance to the organisms. Serine proteases include exopeptidase, endopeptidase, oligopeptidase, and omegapeptidase families(Mamo & Assefa, 2018) **(Figure5)**.

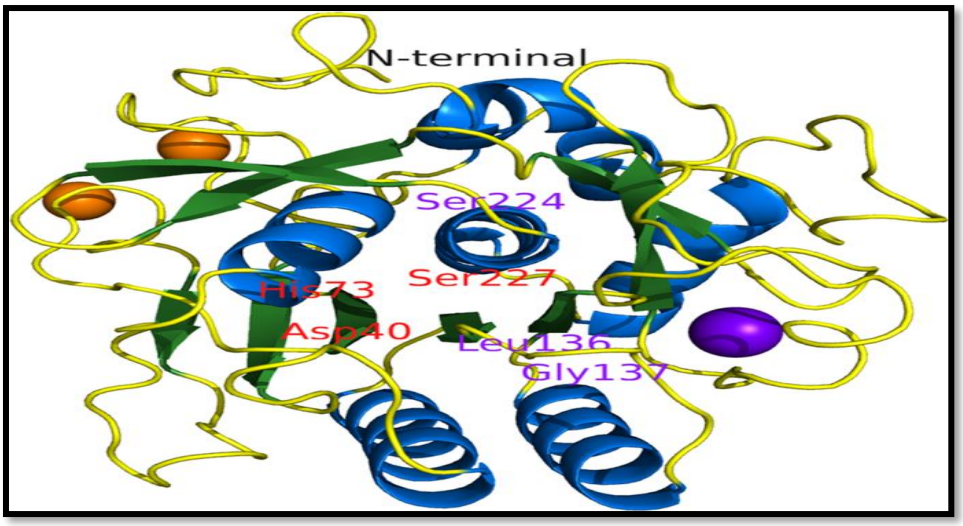

Figure 5: structural model of serine proteases (Li et *al*., 2022).

They are active in both neutral and alkaline pH and have a molecular weight of 18-35 KDa (Ravi Shankar, 2021) and an optimum temperature between 50-70 (Mamo & Assefa, 2018). The catalytic process requires the three residues that make up the catalytic triad: Ser (nucleophile), Asp (electrophile), and His (base) (Ash & Mishra, 2023). According to Ravi Shankar (2021) serine proteases are active in neutral and alkaline pH environments because their best target site cleavage occurs in the pH range of 7 to 11.

2.2.2.5 Cysteine/Thiol proteases

Both prokaryotes and eukaryotes include cyste proteases (EC3.4.22). They are divided into roughly twenty families. Using a catalytic dyad of cysteine and histidine, the activity of all cysteine proteases was assessed. Each family has a different Cys-His or His-Cys residue order **(Figure6)**.

Cysteine proteases often require reducing agents like HCN or cysteine to function. They have maximum activity in the pH range of 2–3 and molecular weight in the range of 34–35 KDa and an optimum temperature between 40–55. Based on their ability to selectively cleave arginine residues, cysteine proteases are categorized into four classes: trypsin-like, papain-like, glutamic acid-specific, and others. Whereas lysosomal proteases act best at an acidic pH, papain cysteine proteases are most active at a neutral pH.

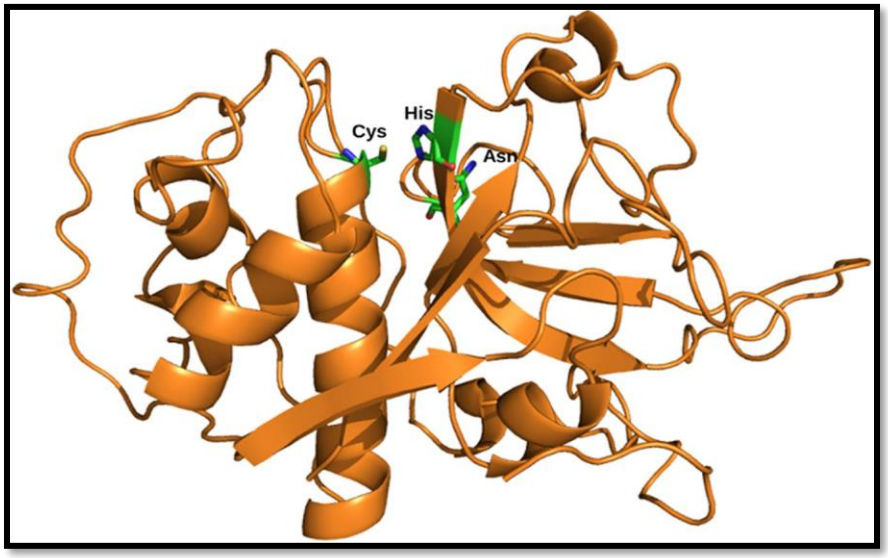

Figure 6: Structural representation of cysteine protease (Verma, 2016)

2.3 Origin of Proteases

Proteases have been found in every known type of organism, including bacteria, plants, and animals. The protease that is secreted is entirely determined by the host and how well suited it is to its surroundings. When producing from various sources, many factors must be carefully taken into consideration (Gurumallesh et *al*., 2019).

2.3.1 Animal Proteases

Renin, chymotrypsin, pepsin and trypsin are the best-known proteases of animal origin. They are produced in large quantities due to their biotechnological potential. However, their production depends on the availability of livestock for slaughter, which is itself governed by agricultural policies (Rao et *al*., 1998).

Chymotrypsin secreted by the pancreas is mainly involved in the digestion of food proteins and also acts as an anti-inflammatory agent by preventing tissue damage (Louati et *al*., 2011). It is used in therapy to accelerate the repair of traumatic, surgical and orthopedic injuries (Shah & Mital, 2018). Pepsin is an acid endopeptidase secreted in the stomach and is involved in protein degradation (Heda et *al*., 2023). It is responsible for damage to the laryngeal epithelium during laryngopharyngeal reflux (Bardhan et *al*., 2012). It is used in the food industry for the production of cheese and to hydrolyze soy allergens (Santos et *al*., 2024).

2.3.2 Plant Proteases

New strategies have been developed over the last decades to reduce the cost and time of producing plant proteases. Generally, these proteases can be obtained by in vitro, in vivo culture or directly from plant biomass using conventional culture. In biotechnology, the best known and most used plant proteases are papain, bromelain, ficin and actinidin.

Papain is extracted from the fruit, root and leaves of the papaya (*Carica papaya*) and is used in the dairy industry, particularly for the production of cheese. While bromelain produced from the stems of pineapple (*Ananas comosus*) is used in meat tenderization and the fish industry. Finally, the actinidine produced by kiwi (*Actinidia deliciosa*) is used to hydrolyze chicken proteins and also for the solubilization of protein aggregates during the production of alcohol (David Troncoso et *al*., 2022)

2.3.3 Microbial Proteases

Two-thirds of the world's commercial proteases are produced by microorganisms such as bacteria, fungi, yeast, and actinomycetes. Microbial proteases are preferred to the proteases from plant and animal sources because they possess all desired characteristics for industrial applications (Devi et *al*., 2008; Razzaq et *al*., 2019).

2.3.3.1 Bacterial Proteases

Bacterial alkaline proteases are characterized by their high activity at alkaline pH between 8 and 12. They have broad substrate specificity with an optimal temperature located around 60°C. These properties make them suitable for use in the detergent industry. For example, Bacillus genera are known for their ability to produce large quantities of alkaline proteases. These proteases are used mainly as additives for detergents and also in several industrial processes such as bioremediation, silk degumming and protein hydrolysate production (Jisha et *al*., 2013).

2.3.3.2 Fungal Proteases

Fungal proteases are also exploited in industry due to their diversity, substrate specificity, and stability under extreme conditions (Jisha et *al*., 2013).

Filamentous fungus generates a variety of hydrolytic enzymes, including proteases. Fungi can produce acid, neutral, alkaline, and metallic proteases. Fungal proteases have broad substrate selectivity and operate across a wide pH range pH 4 -11 (Ash & Mishra, 2023).

2.3.3.3 Yeast Proteases

Yeasts are considered to be the first microorganisms exploited by man. It was used unconsciously by the Sumerians and Babylonians in the production of beer (Oliver, 1991). Nowadays they occupy an important place in biotechnology, particularly in the production of enzymes. Indeed, numerous species of yeast such as *Saccharomyces cerevisiae*, *Candida albic*ans and *Yarrowia lipolytica* have been identified as producers of large quantities of protease (Ogrydziak, 1993). Yeasts produce several types of proteases such as endoproteinases, carboxypeptidases, aminopeptidases and dipeptidylaminopeptidases, located in different compartments and in the cell membrane (Jones, 2002).

Luciana Daniela Lario et al studied the production of proteases by *Rhodotorula mucilaginosa CBMAI 1528*. These proteases are of great interest in biotechnology due to their high activity at low temperatures. This characteristic makes these proteases well suited for use in several

processes requiring exogenous energy input or involving the use of heat-sensitive products (Lario et *al*., 2020).

Clavisora lusitanaie strain is widely exploited in biotechnology for its ability to produce enzymes, particularly acid proteases. The latter proteases are used in enzymatic therapy, particularly in the digestion of gluten. They showed strong activity for the degradation of immunogenic gluten peptides contained in flour and wheat grains. They are therefore considered the best candidates for the treatment of gluten-related diseases (Djekrif et *al*., 2024).

Metschnikowia pulcherrima is also known for the production of proteases which are generally used in wine making to reduce the protein content of grape must. By degrading proteases, these proteases improve the clarification and stabilization of the wine (Vejarano, 2020).

2.4 Application of Proteases

2.4.1 Alkaline Protease

2.4.1.1 Detergent Industry

Alkaline proteases dominate the enzyme market, representing a commercially important group of enzymes and are primarily used as detergent additives. Indeed, the use of alkaline proteases in laundry is more advantageous than traditional detergents. They save energy, time and improve washing efficiency.(Song et *al*., 2023).

2.4.1.2 Medical and Pharmaceutical Industry

Immobilized alkaline proteases from *Bacillus subtilis* are used in therapeutic applications such as ointments composition, non-woven tissues, soft gel medicinal formulas, gauze and new materials. Proteases isolated from *Aspergillus oryzae* helped in diagnostic aid to correct certain enzyme deficiency syndrome. Alkaline fibrinolytic proteases which are used to degrade fibrin have future in anticancer drugs. The elastolytic property of a serine protease from *Bacillus subtilis* has been used to prepare elastoterase. This formulation is used in the treatment of abscesses, burns, carbuncles and other wounds (Pawar et *al*., 2023; Sundus, 2016).

2.4.2 Neutral Proteases

2.4.2.1 Meat Tenderization

Fresh meat pH is neutral, and therefore neutral proteases are best suited for hydrolysis; tenderization of meat is achieved by the action of endogenous proteases, especially neutral lysosomal cathepsins and neutral metalloprotease or cysteine endopeptidase (Song et *al*., 2023).

2.4.2.2 Cosmetic Uses

Neutral proteases are commonly utilized in the manufacture and development of cosmetics. The primary sources of neutral proteases utilized in cosmetics are microorganisms and plants. Tartar from toothpaste can be removed with the aid of neutral protease, most especially *Bacillus subtilis* neutral protease. In order to reduce pathogenic bacteria resistance in the stratum corneum, promote drug absorption, soften the skin, dissolve dandruff, and increase skin metabolism, they are added to the cream (*Site 3*).

2.4.3 Acid Proteases

2.4.3.1 Food Industry

One of the most innovative options for increasing the productivity of the breadmaking business is aspartic proteases, also known as acid proteases. They have the ability to facilitate the absorption of amino acids due to their hydrolytic characteristics. In the process of manufacturing cheese, they are also employed to coagulate milk (Wei et *al*., 2023).

2.4.3.2 Beverage Industry

Acid proteases are also used to digest proteins contained in fruit juices and certain types of alcoholic beverages that make them cloudy, such as blackcurrant, cherry, pomegranate, apple, orange, grape, and kiwi juices (Solanki et *al*., 2021; Song et *al*., 2023) **(Figure7).**

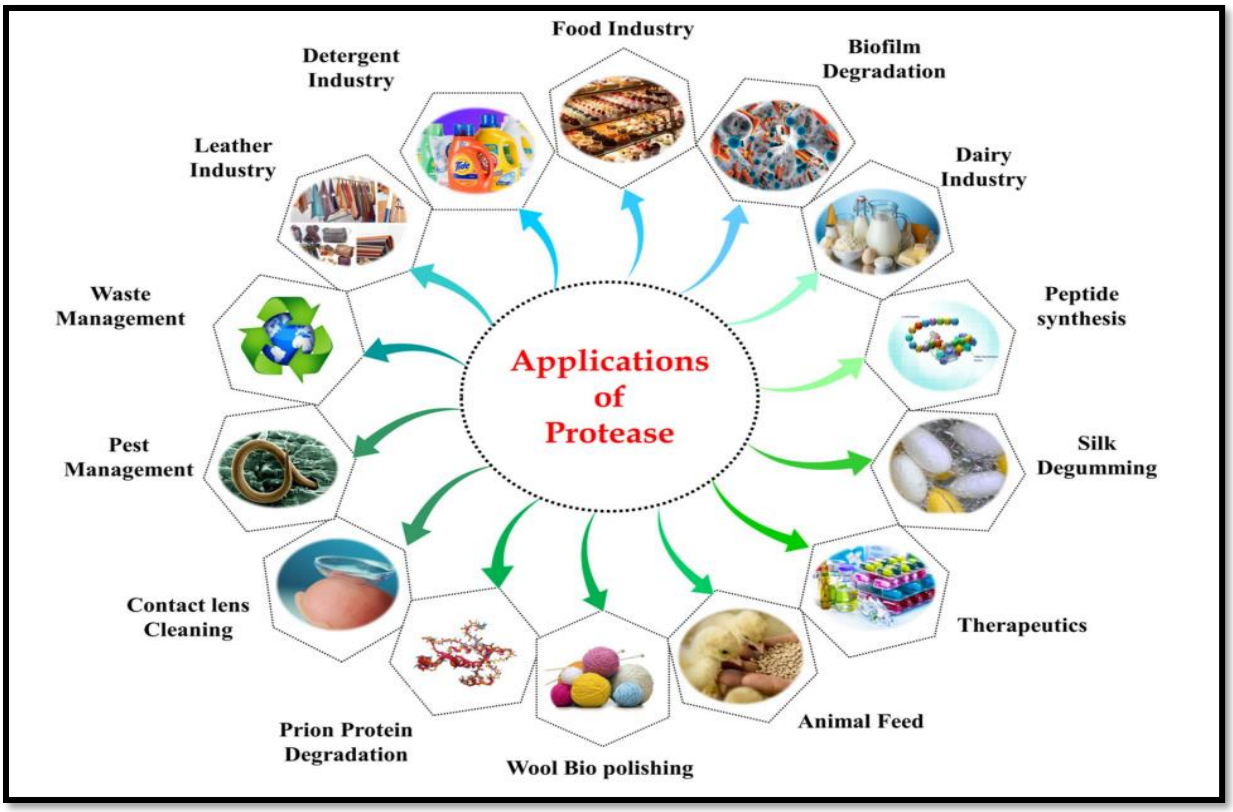

Figure 7: Applications of proteases in various industries/sectors (Solanki et *al*., 2021).

3 Substrates of Proteases

Proteolytic enzymes are more than just catalytic devices that seek for substrates to hydrolyze. As a result, a variety of functional modules or domains that provide substrate specificity, regulate cellular localization, affect kinetic characteristics, and alter susceptibility to endogenous inhibitors are connected to the catalytic domains of many proteases. In terms of specificity, variety is a general norm. Thus, some proteases exhibit exquisite specificity toward a single protein's unique peptide bond (e.g., angiotensin-converting enzyme); however, most proteases are relatively nonspecific for substrates, and some are overtly promiscuous, targeting multiple substrates indiscriminately (e.g., proteinase K) (López-Otín & Bond, 2008). Here are some examples of proteases and their substrates:

- **Thrombin (Serine Proteases):** their substrate is fibrinogen (Plasmatic protein). Where Thrombin cleaves fibrinogen into fibrin during blood clotting.

- **Subtilisins (Serine Proteases):** These are pro-protein convertases. Two examples: furin, located in the Golgi apparatus their substrates among others: MMPs, secretases and parathyroid hormone and pro-protein convertase-1, located in secretion vesicles their substrates among others insulin, glucagon and pro-opiomelanocortin (precursor protein cut into neuropeptides).
- **Renins (Aspartate Proteases):** secreted by the liver, their substrate is angiotensinogen.
- **Matrix Metalloproteinases (MMPs):** A wide range of extracellular matrix proteases (MEC) such as :
  - Collagenases: Their substrate collagen.
  - Gelatinases: Their substrate gelatin.
  - Stromelysin: Theirsubstrates laminin, fibronectin, proteoglycans, collagen -type IV and IX.
  - Matrilysin: Their substrates elastin, fibronectin, proteoglycans.
  - Macrophage el astase: Their substrate elastin (Kramer, 2013.).

1. **Gluten:** a collection of storage proteins present in different cereal grains (Anupam Rej, 2022). Upon ingestion, proteases in the gastrointestinal system breakdown gluten proteins into peptides(Stepniak, 2006).

Chapter three

Materials and methods

4 Materials and Methods

This modest work was carried out at the Microbiological Engineering and Application Laboratory (MEA) department of Biochemistry, Cellular and Molecular Biology, Faculty of Nature and Life Sciences, University of Mentouri brothers, Constantine1. The different stages of our experimental work are as follows:

4.1 Biological Material

4.1.1 Yeast isolation

The yeasts used in our study were isolated from potato peels (PP) (pH 3.9), which are domestic food waste stored in tightly sealed containers **(Figure 8)**.

Figure 8: Potato peels for yeast isolation sampling.

4.1.1.1 Stock solution preparation

To prepare a stock solution, 10 g of potato peel, and Tween 80 at 0.45% added to 90 ml of sterile distilled water. Shake well.

4.1.1.1.1 Dilutions Preparation

After homogenization, prepare a series of decimal dilutions of stock solution up to 10^{-7} (Jerome et *al*., 2004).

-Pour the sterile YGC medium into petri dishes

-After medium solidification, 100 µl of each dilution are spread out separately on the surface of the medium with a rake (Tortora et *al*., 2003).

-Incubate at 30°C for 2-7 days

- Daily observations of the macroscopic and microscopic appearance of the isolates are carried out.

4.1.1.1.2 Medium preparation.

a/ The isolation medium used is YGCA medium (Yeast Extract Glucose Chloramphenicol) g/l **(Appendix 1)**.

b/ The purification and conservation media used are YPGA and YMA **(Appendix 1)**.

- For both media, heat with frequent agitation and boil for one minute to completely dissolve the medium.

-Autoclave at 121°C for 15-20 min.

- Cool to 45 to 50°C and pour into Petri dishes.

- Inoculate the media with yeast

- After inoculation, incubate the plates at 30°C in inverted position (agar side up) with increased humidity.

4.1.1.1.3 Subculture purification of strains

After macroscopic observation, the yeast isolates are subcultured onto YPGA medium (Appendix 1) using the streak method.

- Incubate at 25°C for 3 days. (John and Michael, 2007).

4.2 Conservation of strains

The pure strains are stored on YPGA medium at 4°C and on YPGA with glycerol inclined Medium at -20°C for a year. They were also, stored in cryo-beads at -80°C, for long storage.

4.3 Determination of protease activity

The production of the protease is demonstrated on the skimmed milk agar (SMA) medium:

- 20 g/l of agar (in distilled water) was sterilized in an autoclave at 120°C for 20 min
- Cool to 40°C in a bain-marie
- Add 10% of skimmed milk (Juszczyk et *al*., 2005).
- Inoculate the media with yeast and incubate at 30°C for 4 to 5 days until zones appear.

- The presence of proteolytic enzymes is distinguished by the formation of a clear ring around the colonies reflecting a degradation of caseins in the medium and therefore the presence of proteolytic enzymes (Juszczyk et *al*., 2005).
- Revelation

The enzyme activity is revealed by the appearance of a clear ring-shaped zone surrounding the yeast growth.

- For the protease enzyme, the zone is directly observed without any detector.
- We measured the diameter of the lysis zones for each enzyme and each yeast strain.
  - Low activity: Diameter less than 2 mm
  - Moderate activity: Diameter between 2-10 mm
  - High activity: Diameter greater than 10 mm.

4.4 Yeast Identification

The identification of isolated yeasts, according to conventional methods, is based on the determination of various cultural, morphological, and physiological characteristics (Kurtzman et *al*., 2011).

4.4.1 Cell Shape and Size

To determine the shape and size of yeast cells, microscopic examination is performed on young cultures in YPG and YPGA (24-48 h at 25°C) on a wet mount slide (magnification x40 and/or magnification x100).

4.4.2 Reproduction Mode

- ❖ Asexual reproduction is observed on a wet mount slide (magnification x40 and/or magnification x100). This study is carried out on young cultures in YPGA medium.
- ❖ Sexual reproduction (sporulation):

  The PDA medium is inoculated with the yeast. It is incubated for 7 to 10 days at 25°C. A smear is made on a slide and observed under a microscope in the fresh state (magnification x40 and/or magnification x100).

4.4.3 Filamentation Test

The ability to filament is observed from a culture on PDA medium in a Petri dish. The yeast to be examined is inoculated in a longitudinal streak on the surface of the agar medium **(figure 9)**.

- A sterile coverslip is then placed over the center of the streak. Microscopic observation (magnification x40 and/or magnification x100) is carried out over a period of 3 to 7 days.
- The presence of mycelium and the colony as well as its nature are noted.

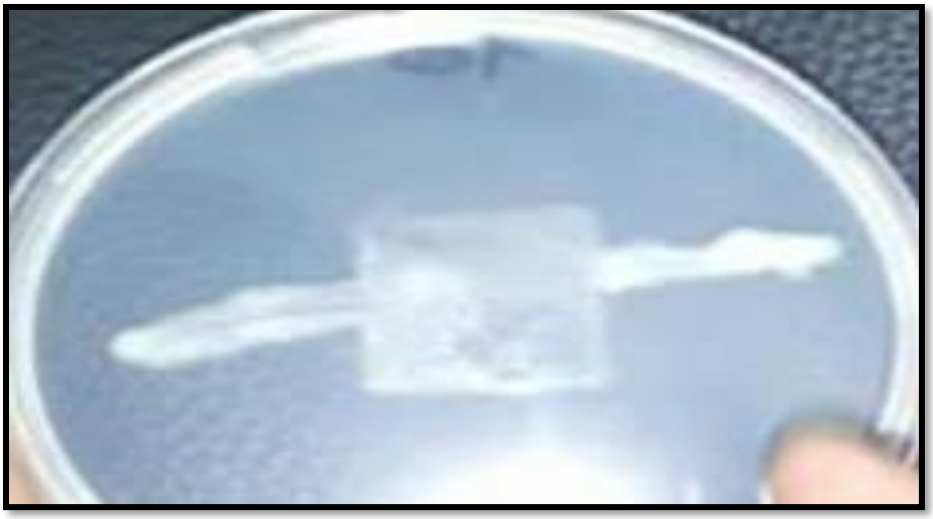

Figure 9: Longitudinal streak on the surface of the agar medium for the filamentation test.

4.4.4 Identification by molecular approach

Yeast strain was submitted for identification via molecular approach (sequencing of the D1/D2 domain of 26S rRNA gene). DNA extraction was carried out according to Bennamoun et al., (Bennamoun et al., 2016) . DNA was first amplified as a template by the PCR method using the primers V9G (50-TGCGTTGATTACGTCCCTGC-30) and RLR3R (50-GGTCCGTGTTTCAAGAC-30; Sigma-Aldrich Co). A 600-650 bp region was sequenced by the forward primer (50-GCATATCAATAAGCGGAGGAAAAG-30) and the reverse primer NL4 (50-GGTCCGTGTTTCAAGACGG-30; Sigma-Aldrich Co). The PCR products were sequenced using a commercial sequencing facility (Macrogen, Amsterdam, Netherlands). The sequences obtained were compared with those included in the GenBank database (Blast freeware from http://www.ncbi.nlm.nih.gov/BLAST) (Bennamoun et *al*., 2016).

4.5 Inoculum Preparation

To prepare the inoculum, 50 ml of YPGA medium is poured into 250 ml Erlenmeyer flasks and then inoculated with P8.

Or PC3 after solidification. After 48 hours of incubation at 30°C, a quantity of sterile distilled water (or physiological saline) is added, and the cells are homogenized by manual agitation. The cell

suspension obtained is stored in the refrigerator at 4C° for few days and used for enumeration and inoculation of the media. Inoculation rate is 10^7 cells/ml.

4.6 Proteases production

4.6.1 Basic medium

Tomato pomace (TP) (collected from restaurants) and the bread waste (BW) (collected from restaurants and bakeries) **(Figure 10)** were selected for the production of proteases, as they have all the nutrients for yeast strains growth.

To determine the content of elements, present in TP and BW, the researchers implemented a set of analytical methods. Both wastes were characterized to determine the following parameters: total sugars (TS) according to the method of Dubois et al., ashes according to the AFNOR method, and fats by the Wisman method and proteins by the Total Kjeldahl method. Nitrogen (TKN). Finally, starch was determined using the iodo-iodide reagent method.

The composition of the tomato was as follows: 22.4% total sugars, 21.2% proteins, 3% lipids and 6.1% minerals and that of the bread was 48.46% total sugars, 46.3% starch, 8.4% protein, 0.28% lipids and 1.3% minerals_ Substrate preparation.

Figure 10: Tomato pomace and Bread Waste.

As part of this research, tomato pomace and bread waste were used (valorized) as an alternative source of carbon and energy for the prod uction of acid proteases by the yeasts

Clavispora lusitaniae and *Rhodotorula mucilaginosa*. Fresh tomato waste was collected and processed to recover only the tomato skin and pulp. Then, they were dried in the open air for 4 days. As for the bread waste, it was cut into small pieces and dried in the open air for 3 days at room temperature. Then they were crushed with blender, then sieved to remove the powder and recover the medium-sized particles (2 mm). Both wastes are kept in tightly closed boxes (Djekrif et *al*., 2024).

4.6.2 Fermentation

The fermentation was carried out on a solid-state fermentation (SSF), based on TP and BW prepared previously. For production, six 250 ml Erlenmeyer flasks were prepared. In each Erlenmeyer flask, the two substrates were introduced in equal quantities (5g of TP and 5g of BW). Then a quantity of distilled water was added to obtain a humidity of 60%. The contents of each Erlenmeyer flask were carefully mixed using a glass rod. Then, the Erlenmeyer flasks were sealed using carded cotton and aluminum foil and were placed in an autoclave for sterilization.

4.6.3 Selection of microbial culture type

Protease production was studied in monoculture of PC3 and P8 and in mixed culture of both strains. All cultures are incubated at 30°C in an oven for 48 hours.

4.6.4 Selection of protease type

For selection of protease type, the enzyme activity from PC3 and P8 was assayed at different pH: 4, 7 and 9.

4.7 Production Optimization

The improvement of the production environment was done with the aim of increasing the production of protease by examining the effect of factors on enzymatic production, namely: glucose, sucrose, yeast extract, corn steep liquor, Na NO_3, $MgSO_4$ and KH_2PO_4. For this, the use of the statistical designs of Plackett and Burman (1946) was chosen, they allow the screening and selection of factors with a significant positive or negative effect.

4.7.1 Plackett and Burman's design (PBD)

The Plackett and Burman matrices provide an efficient method for selecting a large number of variables and identifying the most important ones. These designs have shown their effectiveness in the study of the optimization of microbial enzyme production. They have already been used in other works for the selection of factors influencing the production of milk coagulating enzymes in

Bacillus amyloliquefaciens(Zhang et *al*., 2013), β-galactosidase in Talaromyces pinophilus (El-Naggar et *al*., 2015), and α-amylase from Bacillus amyloliquefaciens KCP2 (Prajapati et *al*., 2015).

These plans make it possible to highlight the effect of k factors at two levels (Table) on the enzymatic production studied with a minimum number of experiments N such that N = k + 1 (Zhang et *al*., 2013). These are square matrices established from a base generator, with the last line of the experimental design always being at the lower level. Each row represents an experiment, and each column represents a variable.

In our study, the matrix is composed of 11 factors (12 experiences) of which 4 are designated as error variables in order to maintain a statistical reserve for the evaluation of the experimental error **(table 2).**

Table 2: Plackett–Burman design for the study of 11 factors with 12 experiments.

Trials	Variables										
	A	B	(C)	D	E	(F)	G	H	(I)	J	(K)
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

C, F, I and K are the error variables: +, higher level; -, lower level

The number of real variables is therefore reduced to N-4, then the study of 7 factors which are glucose, sucrose, yeast extract, corn steep liquor, Na NO_3, $MgSO_4$ and KH_2PO_4. The different levels of each variable are recorded in Table **(Table 3)**.

Table 3: Correlation between the coded levels and the real levels of the factors studied in the design of Plackett and Burman.

Factors	Level		Unit
	−1	**+1**	
A: Glucose	0	1	%
B: Sucrose	0	1	%
C: Error	**-**	**-**	**-**
D: Yeast Extract	0	2	%
E: Corn Steep Liquor	0	0.2	ml/L
F: Error	**-**	**-**	**-**
G: $NaNO_3$	0	0.3	%
H: $MgSO_4$	0	0.1	%
I:Error	**-**	**-**	**-**
J: K_2HPO_4	0	0.1	%
K: Error	**-**	**-**	**-**

Matrix processing is carried out by Minitab 19; the statistical analysis consists of identifying the variables which have a significant positive or negative effect on the production of protease.

In order to carry out this experiment, 24 Erlenmeyer flasks of 250 ml; 12 Erlenmeyer flasks for each of both strains are cultured in a 50% bread waste (2.5g) and a 50% tomato Pomace (2.5g) culture medium. After humidification, the media are supplemented according to the matrix (table); After sterilization at 120°C for 20 min, they are inoculated with 10^7 cells/ml of the corresponding strain and incubated at 30°C for 48 hours.

4.8 Enzymatic extraction

Once the incubation period is ended, 50% (2.5g) of fermented substrates from each Erlenmeyer flask are weighed in glass Petri dishes (Pre-measured) and incubated in an oven at 70°C until dry weight to determine biomass.

Furthermore, the remaining 50% of fermented substrate was mixed with 12.5 mL of Tween 80 solution (0.02%) and shake for 3 minutes using a vortex mixer. the solution is subsequently centrifuged at 10,000× g at 4 °C for 10 min. The supernatant was filtered using Whatman paper n°1, and the filtrate was used as a crude enzyme.

4.9 Analytical methods

4.9.1 Protease activity Assay

The proteolytic activity in enzymatic extract of *Clavispora lusitaniae PC3 and Rhodotorula mucilaginosa P8* was measured according to method described by Benkahoul et *al*., (2020) (Benkahoul et *al*., 2020) with minor modifications using casein as substrate. The mixture containing 0.625 ml of 2.5% casein as the substrate and 0.25 ml of enzymatic extract and 0.375 ml of buffer was prepared. It was incubated at 40°C for 30 min, and then 2.5 ml of trichloroacetic acid (TCA) 4% were added to the reaction mixture to terminate the enzyme reaction. After 10 min, the reaction mixture was filtrate and to 0.5 ml of filtrate, 2.5 ml of Na2CO3 2% and 0.25 ml of 25% Folin Ciocaltreu reagent (before use, 25 ml of reagent diluted with 100 ml of distilled water) were added and the mixture was incubated at room temperature. After 30 min, the protease activity was read at 750 nm in a spectrophotometer (UV/VIS) using a tyrosine standard curve as a reference **(Appendix 2).** Simultaneous controls containing enzyme, heat-denatured enzyme and substrate were maintained. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmole tyrosine/min.

4.10 Proteases selection

To determine which protease is most produced by our cultured strains, we assayed protease activity at three pH (4, 7 and9): In this experiment, the buffer solutions are prepared: Citrate-phosphate buffer for pH 4 and pH 7, and glycine-NaOH buffer for pH 9.

4.11 Confirmation of the type of protease produced by the strains

The type of protease produced by both *Clavispora lusitaniae* and *Rhodotorula mucilaginosa* is confirmed by the determination of the optimum pH and the study of the effect of pepstatin (1µg/ml) on the protease activity.

Chapter four

Results and discussion

5 Results and Discussion

5.1 Isolation and Identification of Yeast Isolate

Proteases are enzymes with numerous industrial applications. To meet the increasing demand for proteases with specific properties, researchers are exploring new sources. In our study, two yeast strains (PC3 and P1) were selected for in-depth analysis. For this purpose, three tests were conducted: macroscopic and microscopic observations, sporulation test, and filamentation test. The results of these tests are recorded in Table 4.

Table 4: Identification from biochemical (ID 32C), microscopic and molecular biology characters.

Strain code	macroscopic observation	microscopic observation	Filamentation	sporulation	**strain**	**Accession number**
PC3	Smooth, glistening, butyrous, White to cream colored, entire margin.	Sub-globose, ovoid to elongate Budding (unipolar and bipolar)	Pseudomycelium	+	*Clavispora lusitaniae*	PP057739
P8	Pink, smooth/glossy, mucoid, convex elevating, entire margin	Subglobose, ovoid to elongate	-	-	*Rhodotorula mucilaginosa*	PP892316

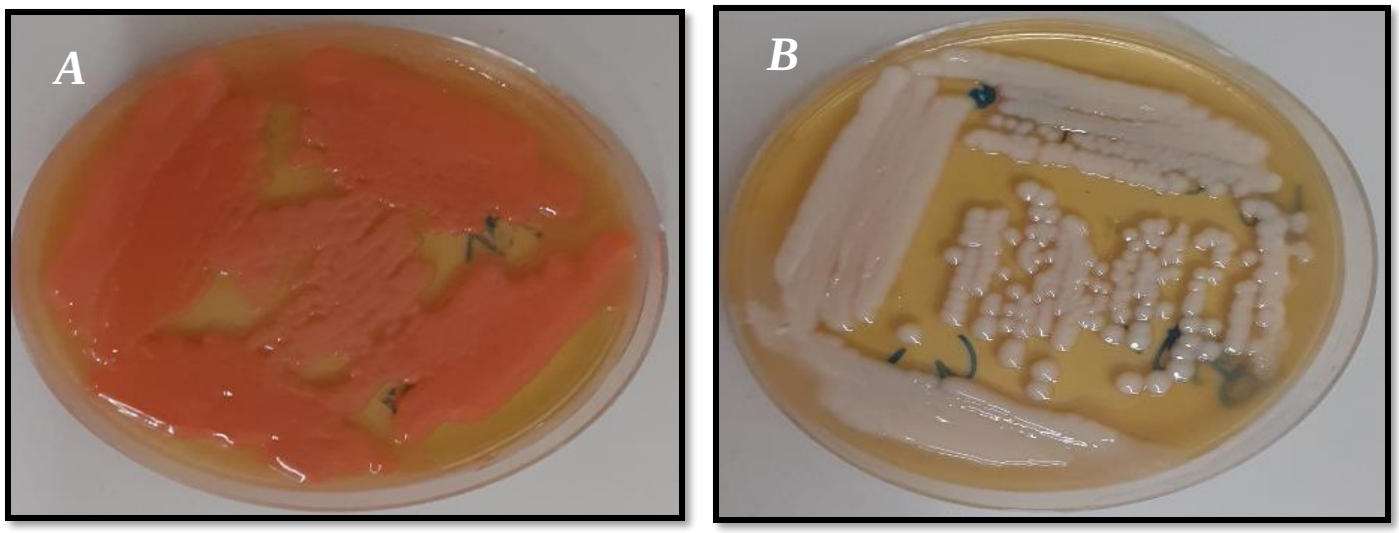

Figure 11*: A :*Rhodotorula mucilaginosa* Strain on YPGA, **B :** *Clavispora lusitaniae* Strain on YPGA

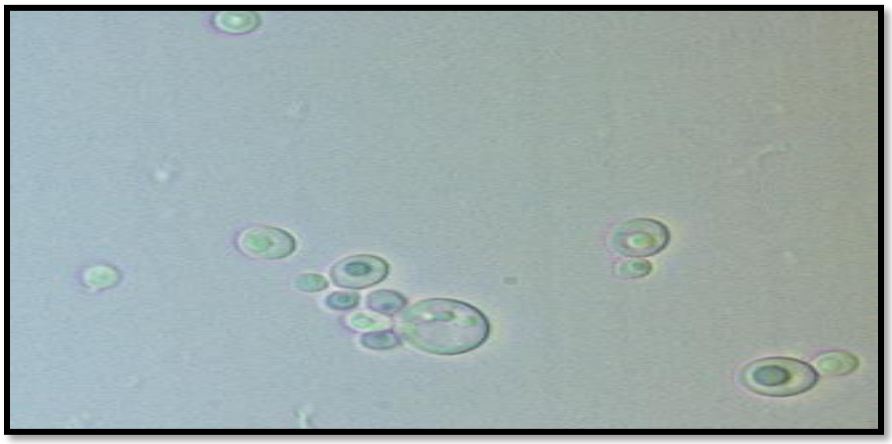

Figure 12: sporulation in *clavispora lusitaniae.*

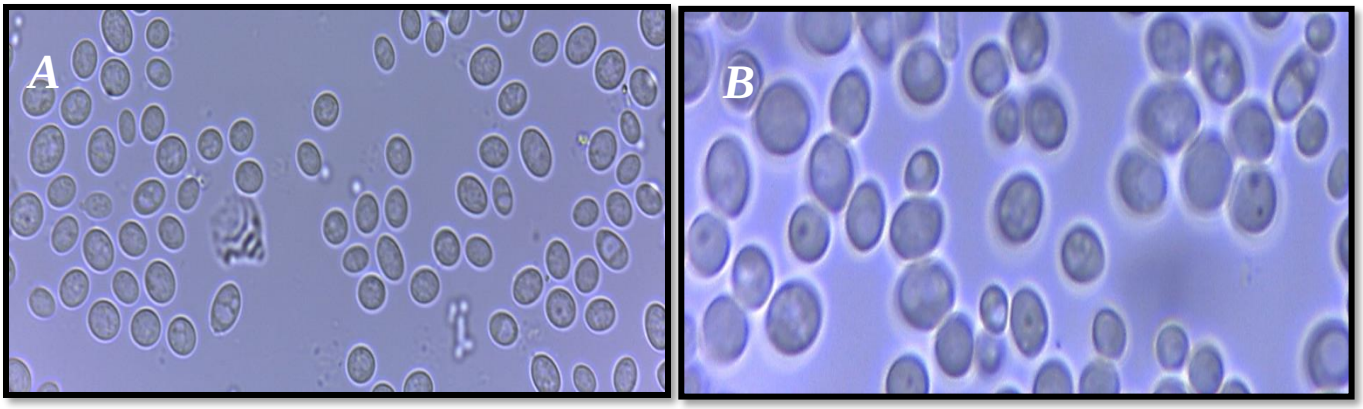

Figure 13: Microscopic aspect of A: *Rhodotorula mucilaginosa* and B: *Clavispora lusitaniae*

The selected yeast strain was submitted for identification via molecular approach:

The yeast Clavispora lusitaniae was identified using ITS sequencing with the following sequence:

>Clavispora_lusitaniae_ITS

TTCTAACAAACTAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAG
AACGCAGCGAATTGCGATACGTAGTATGACTTGCAGACGTGAATCATCGAATCTTTGAAC
GCACATTGCGCCTCGAGGCATTCCTCGAGGCATGCCTGTTTGAGCGTCGCATCCCCTCTA
ACCCCCGGTTAGGCGTTGCTCCGAAATATCAACCGCGCTGTCAAACACGTTTACAGCACG
ACATTTCGCCCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGA
GGAAAAGAAACCAACAGGGATTGCCCCAGTAACGGCGAGTGAAGCGGCAAAAGCTCAA
ATTTGAAATCCTGCGGGAATTGTAATTTGAAGGTTTCGTGGTCTGAGTCGGCCGCGCCCA
AGTCCATTGGAACATGGCGCCTGGGAGGGTGAGAGCCCCGTATGGCGCACGCCGACTCT
TTGCACCGCGGCTCCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAA
TTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAA
GATGAAAAGCACTTTGAAAAGAGAGTGAAACAGCACGTGAAATTGTTGAAAGGGAAGG
GCTTGCAAGCAGACACGGTTTTACCGGGCCAGCGTCGA

- Similarly, *Rhodotorula mucilaginosa* was identified with the following ITS sequence:

>Rhodotorula_mucilaginosa_P8_ITS

CGAACTCCTATTCACTTATAAACACAAAGTCTATGAATGTATTAAATTTTATAACAAAAT
AAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCG
ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCT
CCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGTCATGAATACTTCAACCCTCCTCTTTC
TTAATGATTGAAGAGGTGTTTGGTTTCTGAGCGCTGCTGGCCTTTACGGTCTAGCTCGTT
CGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTTGGCGTAATAGACTATTCGC
TGAGGAATTCTAGTCTTCGGACTAGAGCCGGGTTGGGTTAAAGGAAGCTTCTAATCAGAA
TGTCTACATTTTAAGATTAGATCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATA
TCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAGCGGCGAGCGAAGCGGGAAG
AGCTCAAATTTATAATCTGGCACCTTCGGTGTCCGAGTTGTAATCTCTAGAAATGTTTTCCGCGTT
GGACCGCACACAAGTCTGTTGGAATACAGCGGCATAGTGGTGAGACCCCCGTATATGGTGCGGA
CGCCCAGCGCTTTGTGATACATTT

5.2 Protease production

5.2.1 Culture selection

For the production of proteases, two yeast strains, *Clavispora lusitaniae* and *Rhodotorula mucilaginosa*, were studied in monoculture and mixed culture in solid-state fermentation using a

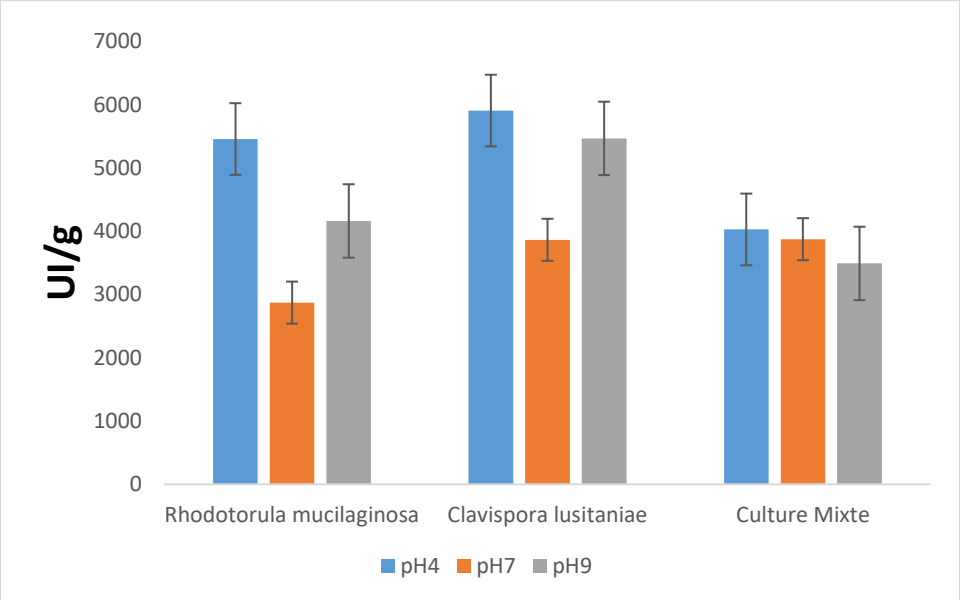

Figure 14: Production of *Clavispora lusitaniae PC3* and *Rhodotorula mucilaginosa P8* protease on different solid media.

TP and BW mixture as substrate. The obtained results are presented in the **figure 14**.

According to the results shown in the figure, the best enzymatic production was observed in monoculture media of *Clavispora lusitaniae* (5909.055 ± 937 UI\g) and *Rhodotorula mucilaginosa* (5458.85 ± 2027 UI\g). However, the mixed culture resulted in low production (4029.58 ± 576 UI\g).

It is important to note that interactions between microorganisms in mixed culture systems can have positive or negative effects on growth and biomolecule production. In our case, the low production of proteases in the mixed culture may be explained by the synthesis of primary or secondary metabolites by one or both yeast strains, which have an inhibitory effect on protease production (Bader et *al.*, 2010). In addition, other studies mention the possibility of severe compensations between microorganisms for nutrient, oxygen and space consumption (Hétian Hu, 2021).

5.2.2 Protease Selection

Fermentation of the two yeasts in monoculture and mixed culture was conducted using solid-state fermentation on a substrate consisting of TP (pH = 4.2) and BW (pH = 5.4). For each culture, protease activity was studied at three pH levels: pH 4, pH 7 and pH 9.The results of protease production are presented in the following **figure 15.**

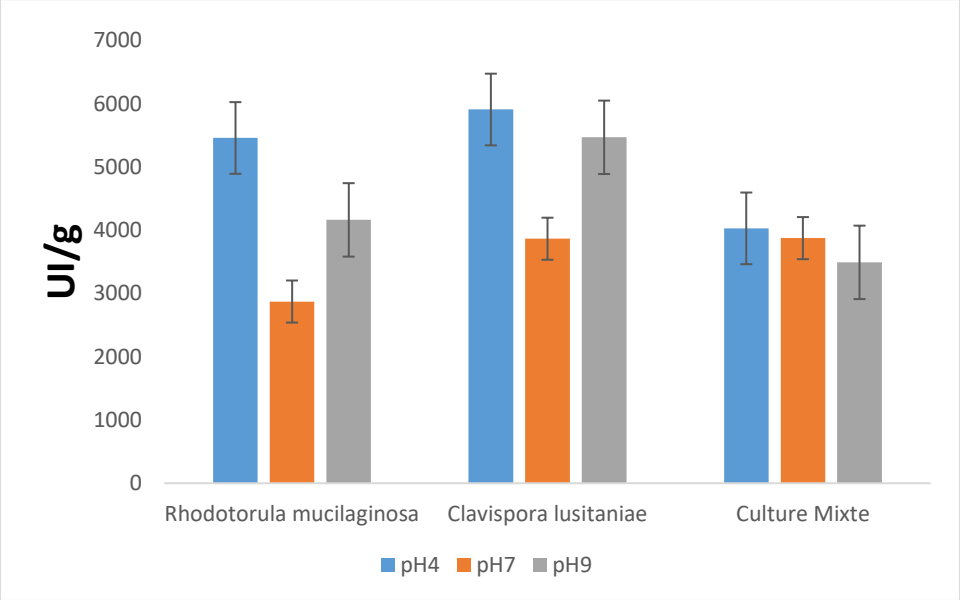

Figure 15: Production of *Clavispora lusitaniae PC 3* and *Rhodotorula mucilaginosa P8* protease on diferent pH levels.

From the results, it appears that for the 3 cultures in particular, monoculture of *Clavispora lusitaniae*, *Rhodotorula mucilaginosa* and the mixed culture, the best enzymatic production was shown at pH 4 (5909.055 ± 937 UI\g ; 5458.85 ± 2027 UI\g ; 4029.58 ± 576 UI\g, Respectively)

The results in **Figure 15** indicate that the acidic environment is well-suited for the growth of both strains, *Clavispora lusitaniae* and *Rhodotorula mucilaginosa*, and allows good production of protease at a pH of 4. On the other hand, the absence of production of alkaline and neutral proteases can be attributed to the unfavorable pH of the environment. Indeed, the synthesis of proteases is influenced by pH, which regulates their yield and determines the type of protease produced (Abdelal et *al*., 1977).

Similar results were also obtained by Rucha et *al*. (Rüchel et *al*., 1982). In their study on protease production by *C. albicans*. They found that the strain produced acidic protease activity, but not neutral or alkaline protease activity when grown in an acidic medium

Therefore, neutral or alkaline proteases might not be produced in an acidic environment unless the pH of the medium is adjusted during fermentation. This is reflected in the results obtained by James(James, 2004) , who found catalytic activity of carboxypeptidase enzymes in a pH range of 6 to 9.

5.3 Optimization of Protease Production from *PC3* and *P8*

5.3.1 Screening of Significant Factors

The optimization of protease production in *Clavispora lusitaniae* and *Rhodotorula mucilaginosa* was carried out using the Plackett-Burman statistical design. We used a matrix of 12 trials for 7 real factors and 4 error factors. The results are presented in the following table:

Table 5. Plackett–Burman's design for the study of 11 factors with 12 experiments with the prtease activity.

Experiments	**A**	B	C	(D)	E	F	G	(H)	I	J	(K)	*Clavispora lusitaniae* Protease activity (IU/g)	*Rhodotorula mucilaginosa* Protease activity (IU/g)
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	12058.06	11753.42
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	12437.5	12476.82
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	13882.75	12952.7
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	13089.04	9715.23
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	11843.13	11306.12
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	2208.52	10266.66
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	12425.67	12475.86
8	+1	+1	-1	-1	-1	-1	+1	-1	+1	-1	+1	12534.24	11900.66
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	13062.5	10398.64
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	13815.78	14610.39
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	14048.27	12745.09
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	14290.54	11310.19

A, B, C, E, F,G,I and J are real variables

D, H and K are dummy variables

To evaluate the effect, we used Student's t-test by manipulating the response when the factor varies between its lower level (-1) and its upper level (+1). We also evaluated the p-value for each factor. Factors with a p-value less than 0.05 (p-value < 0.05) were considered significant for protease production(Dakhmouche Djekrif et al., 2024).

The statistical analysis of the results was performed using Minitab 19 software, and the analyses are presented in the **table 6**.

Table 6: Statistical study of the effect of tested factors on the production of acid protease of the two strains.

souche	Terme	Coeff	Coef ErT	Valeur de T	Valeur de p	FIV	souche	Terme	Coeff	Coef ErT	Valeur de T	Valeur de p	FIV
P8	Constante	1468,1	10,6	138,65	0,000		PC3	Constante	1580,6	19,0	83,20	0,000	
	C1	37,1	10,6	3,50	0,025	1,00		C1	59,0	19,0	3,11	0,036	1,00
	C2	-12,7	10,6	-1,20	0,297	1,00		C2	21,8	19,0	1,15	0,315	1,00
	C4	39,6	10,6	3,74	0,020	1,00		C4	80,6	19,0	4,24	0,013	1,00
	C5	-77,9	10,6	-7,36	0,002	1,00		C5	-9,4	19,0	-0,49	0,647	1,00
	C7	-9,5	10,6	-0,90	0,421	1,00		C7	-88,2	19,0	-4,64	0,010	1,00
	C8	32,8	10,6	3,10	0,036	1,00		C8	-73,0	19,0	-3,84	0,018	1,00
	C10	-23,3	10,6	-2,20	0,092	1,00		C10	34,6	19,0	1,82	0,143	1,00

Based on the results on the Table, Protease production was influenced by factors with a significant positive or negative effect ($p \leq 0.05$). For *Rhodotorula mucilaginosa*, A: Glucose (p = 0.025), D: Yeast Extract (p = 0.020), and H: MgSO4 (p = 0.036) have a significant positive effect on protease production, while E: Corn Steep Liquor (p = 0.002) has a significant negative effect. As for *Clavispora lusitaniae*, the factors A: Glucose (p = 0.036), D: Yeast Extract (p = 0.013) have a significant positive effect on protease production, while, G: $NaNO_3$ (p = 0.010), H: $MgSO_4$ (p = 0.018) have a significant negative effect.

- **Reduced equation**

The reduced polynomial equation for protease production (Y) for the two strains:

- ✓ Y (*Rhodotorula mucilaginosa*) = 1468.1 + 37.1 Glucose + 39.6 Yeast Extract – 77.9 Corn Steep Liquor

- ✓ Y (*Clavispora lusitaniae)* = 1580.6 + 59.0 Glucose + 80.6 Yeast Extract – 88.2 $NaNO_3$ – 73.0 $MgSO_4$

➢ **Regression equation**

The results of the statistical analysis of the PBD showed that the proteolytic activity could be presented by the following regression equation:

- ✓ *Rhodotorula mucilaginosa:* C12 = 1468, 1 + 37, 1 C1 - 12, 7 C2 + 39, 6 C4 - 77, 9 C5 - 9, 5 C7 + 32, 8 C8 - 23, 3 C10.
- ✓ *Clavispora lusitaniae:* C12 =1580,6 +59,0 C1 + 21,8 C2 + 80,6 C4 – 9,4 C5 – 88,2 C7 – 73,0 C8 + 34,6 C10.

The statistical analysis indicates a coefficient of determination R^2=96.04% for *Rhodotorula mucilaginosa* for this model. This means that 96.04% of the variance in acid protease production is explained by this model, while the remaining 3.87% is unexplained. As for Clavispora lusitaniae, the coefficient of determination R^2=94.51%. For this model. This means that 94.51% of the variance in acid protease production is explained by this model, while the remaining 5.49% is unexplained **(table 7)**.

Table 7. Model summary of P8 and PC3

P8	**S**	**R carré**	**R carré (ajust)**	**R carré (prév)**	**PC3**	**S**	**R carré**	**R carré (ajust)**	**R carré (prév)**
	36,6808	96,04%	89,11%	64,36%		65,8106	94,51%	84,90%	50,58%

➢ **Pareto charts**

For *Rhodotorula mucilaginosa* The Pareto chart **(Figure 16)** graphically represents the results of the experimental design for protease production, illustrating the order of significance of the Variables. It is clear from the chart that Glucose (C1), Yeast Extract (C4), Corn Steep Liquor (C5) and $MgSO_4$ (C8) significantly affect protease production, and the quadratic term is indicated as 2.776.

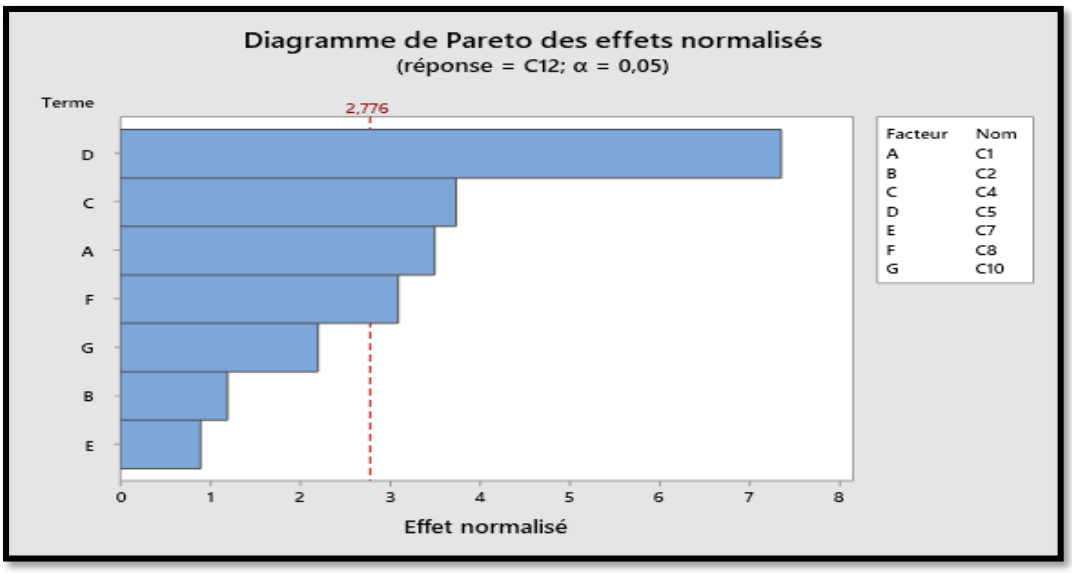

Figure 16: Pareto chart of standardized effects of operational parameters on protease activity of *Rhodotorula mucilaginosa*.

For *clavispora lusitaniae* The Pareto chart **(Figure 17)** graphically represents the results of the experimental design for protease production, illustrating the order of significance of the variables. It is clear from the chart that Glucose (C1), Yeast Extract (C4), $NaNO_3$ (C7) and $MgSO_4$ (C8) significantly affect protease production, and the quadratic term is indicated as 2.776.

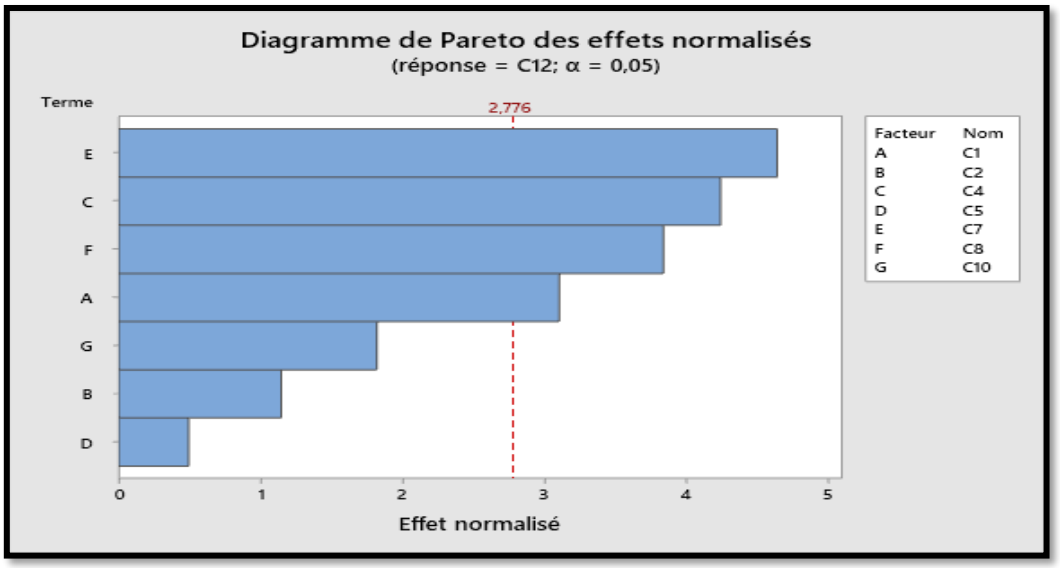

Figure 17: Pareto chart of standardized effects of operational parameters on protease activity of *Clavispora lusitaniae.*

In conclusion, the design enables the selection of factors that have either a positive or negative effect on protease production in both strains.

5.3.2 Model validation

The factors having been determined, a production trial under these conditions (Glucose, Yeast Extract, Corn Steep Liquor and $MgSO_4$) for *Rhodotorula mucilaginosa* and the conditions (Glucose, Yest Extract, $NaNO_3$ and $MgSO_4$) for *Clavispora lusitaniae* was conducted using solid-state fermentation on a culture medium consisting of 50% TP and 50% BW.

The optimization of the culture medium resulted in an approximately three-fold increase in protease activity for both *Clavispora lusitaniae* and *Rhodotorula mucilaginosa* compared to the activity of the initial production medium (5909.055 ± 937 U/g and 18421.88 ± 662 U/g**;** 5458.85 ± 2027 U/g and 17865.17 ± 794 U/g respectively).

In this study, PBD proved effective in optimizing enzyme production. The cost of production media has always been a primary concern due to the commercial interest in the enzyme (Chaud et al., 2016). Protease production by *Cl. lusitaniae PC3* and *Rh.mucilaginosa P8* in the initial medium was deficient compared to the optimized medium. Therefore, we conclude that optimizing the initial culture medium demonstrated the potential to increase yeast protease production and reduce the culture medium cost by utilizing food waste.

5.4 Protease Characteristics

5.4.1 Effect of pH on Protease Activity

Different pHs were studied on the protease activity of *Rhodotorula mucilaginosa and Clavispora lusitaniae*.

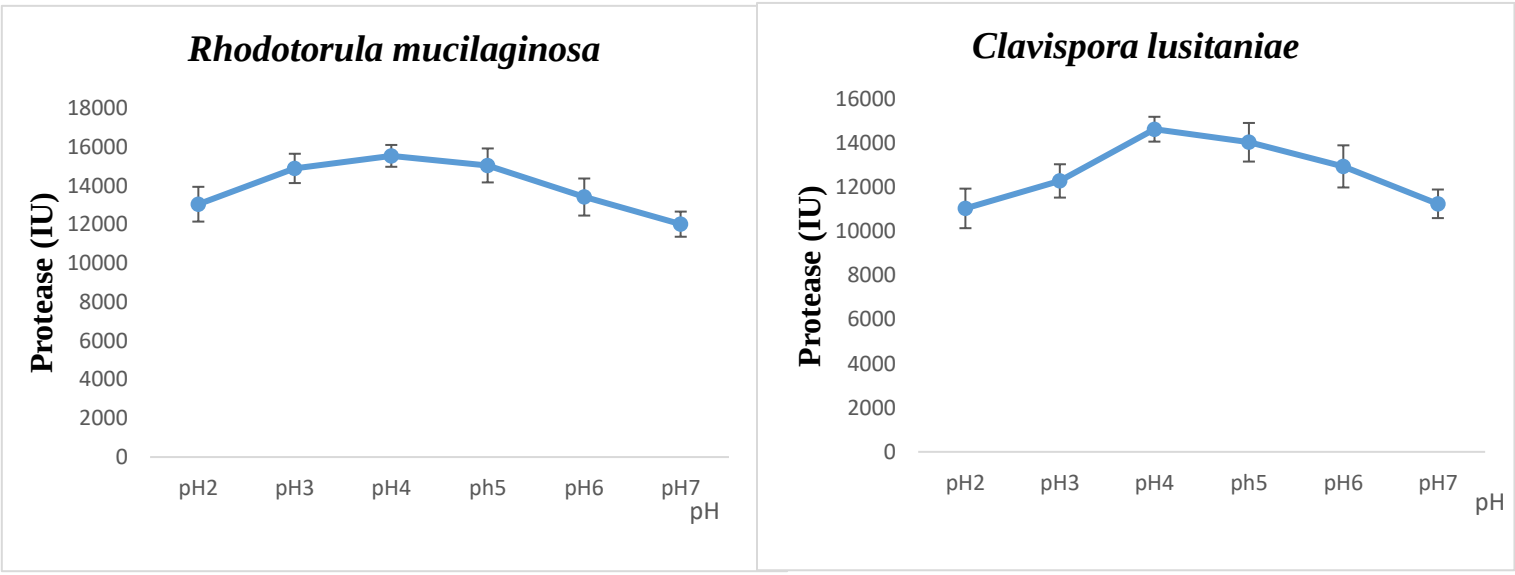

Figure 18: Effect of pH on Protease Activity of *Rhodotorula mucilaginosa P8* and *clavispora lusitaniae PC3* proteases.

The results in the **figure 18** shows that protease activity increases with pH up to the optimal pH (pH 4) beyond which the activity decreases.

Enzymes have a wide range of electrolytic sensitivity to various pH values and other conditions *Rhodotorula glutinis, Rhodotorula parapsilosis, Rhodotorula tropicalis, Rhodotorula humicola, Rhodotorula mucilaginosa L7, and Rhodotorula oryzicola* all produced extracellular acidic protease, with optimal pH values of 2.8 to 3.5, 4.3, 3.4 to 3.8, 4, 5, and 6.51, respectively.(Dakhmouche Djekrif et *al*., 2024; Lario et *al*., 2015; Ray et *al*., 1992)

5.4.2 Effect of pepstatin on protease

The effect of pepstatin on the protease of *Rhodotorula mucilagenosa* and *Clavispora lusitaniae* was studied and the result is shown in the **figure 19.**

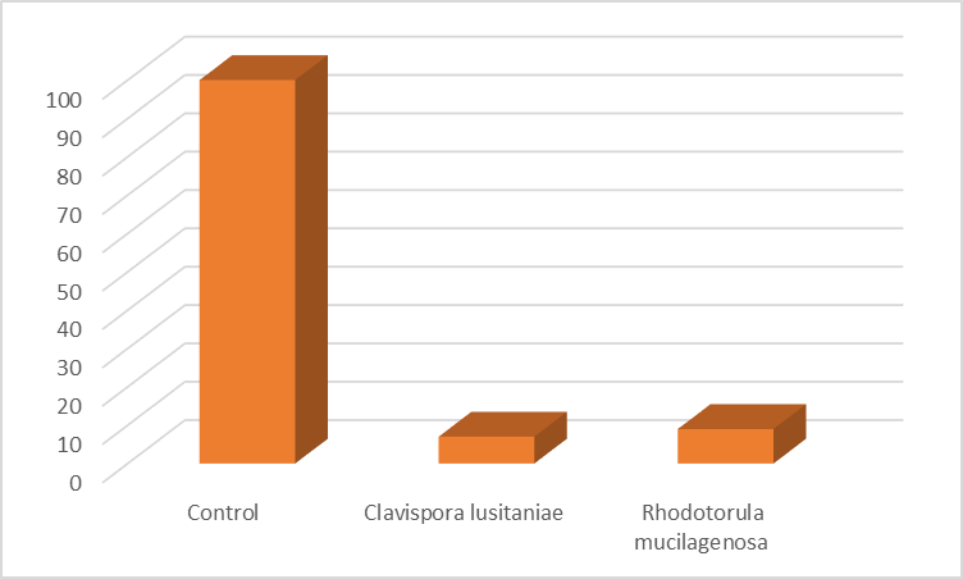

Figure 19: effect of pepstatine on poteases activity from *Rhodotorula mucilaginosa* and *Clavispora lusitaniae.*

According to these results, the optimal pH for the activity of the protease from *Rhodotorula mucilagenosa* and *Clavispora lusitaniae* is pH 4. And pepstatin inhibited the protease activity. This allows us to conclude that protease from *Rhodotorula mucilagenosa and Clavispora lusitaniae* is an acidic protease.

General conclusion

General conclusion

This thesis presents a comprehensive study on the isolation, selection, optimization, and characterization of yeast strains for protease production from agro-industrial waste.

In the first part, *Clavispora lusitaniae* and *Rhodotorula mucilaginosa* strains were isolated and identified from potato waste for their ability to produce protease. For protease production, the two strains were studied in monoculture and mixed culture using solid-state fermentation on a substrate of tomato waste and bread residues. The results show that monocultures (5909.055 UI\g for *clavispora lusitaniae* and 5458.85 UI\g for *Rhodotorula mucilaginosa)* are better than mixed cultures ((4029.58 UI\g). However, the yeasts grown on waste produced good results. Our study contributes to minimizing environmental pollution, which is an important point. Not only were the strains isolated from waste, but the production was also carried out in a culture medium based on waste, thus contributing to reducing environmental pollution and producing a high-value metabolite: protease. This enzyme, widely used in various industries, accounts for 60% of the global protease market. Producing this protease in Algeria, in local laboratories, using local strains and an inexpensive fermentation medium based on agro-food waste, presents significant potential for local industrial development.Protease activity was tested at three different pH levels (4, 7, 9), revealing that the strains produce all three types of proteases (acid, basic and neutral), with acidic proteases being the most dominant with an activity of (5909.055 UI\g for *clavispora lusitaniae* and 5458.85 UI\g for *Rhodotorula mucilaginosa*).

In the second part, the optimization of protease production was carried out using the Plackett-Burman design to select the factors having a significant effect on protease production, namely a Glucose of 1%, Yeast Extract of 2%, Corn Steep Liquor of 0.2 ml/L and $NaNO_3$ of 0.3%, and the activity approximately three times higher than the initial for both *Clavispora lusitaniae* and *Rhodotorula mucilaginosa* (5909.055 ± 937 U/g and 18421.88 ± 662 U/g**;** 5458.85 ± 2027 U/g and 17865.17 ± 794 U/g respectively).Moreover, pH characterization indicated that the proteases from both strains are acidic and can be inhibited by pepstatin, confirming that the produced proteases are acidic.

This thesis proposes the creation of a solid-state fermentation (SSF) process for the production of an enzyme of technological value. The SSF technique has been shown to be effective in recycling industrial tomato waste and bread residues to develop a low-cost acidic yeast protease.

General conclusion

Finally, these results require further studies and open new perspectives:

- Study of Growth and Production Kinetics: It will be interesting to study the kinetics of yeast growth and protease production.
- Protease Production Kinetics *of Clavispora lusitaniae and Rhodotorula mucilaginosa*: Investigate the kinetics of protease production by *Clavispora lusitaniae PC3* and *Rhodotorula mucilaginosa P8*.
- Purification and Characterization: Conduct complete purification and use various chromatography techniques (gel filtration, ion exchange, HPLC) and electrophoresis.
- Industrial Application Trials: Carry out trials for the industrial application of the enzyme.
- Optimization of Fermentation Conditions: Further optimize fermentation conditions to enhance enzyme yield and activity.
- Enzyme Stability and Efficiency: Assess the stability and efficiency of the proteases under different industrial conditions.
- Genetic Engineering Approaches: Explore genetic engineering techniques to improve the strains and increase protease production efficiency.
- Evaluation of Alternative Substrates: Investigate the use of other food waste substrates to diversify and potentially improve protease production.

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Appendixes

Appendix 1

1. The isolation medium YGCA medium (Yeast Extract Glucose Chloramphenicol) g/l:
   - Yeast extract
   - Glucose
   - Chloramphenicol to prevent bacterial contamination.
   - Agar
2. The purification and conservation media YPGA and YMA:

➢ **YPGA**

Dissolve in 1l of distilled water:

- Peptone
- Yeast extract
- Glucose
- Sodium chloride
- Agar

➢ **MA**

Dissolve in 1 l of distilled water:

- Peptone
- Yeast extract
- Sodium chloride
- Glucose
- K2HPO4
- Agar

3. **Yeast Extract Peptone Glucose Agar (YPGA)**

This is the previous medium, 2% agar. Dissolve in 1 liter of distilled water:

20 g glucose.

10 g of peptone.

5 g of yeast extract.

By shaking the mixture on a stirrer until complete dissolve of all the ingredients. After adding 20 g of agar (or 4 g for each flask of 200 ml). The medium is autoclaved, the distribution of the culture medium in Petri dishes is performed in a sterile area in front of a Bunsen burner to avoid contamination of the environment.

Appendix 2

1. Tyrosine calibration curve

The standard range is from a stock solution of tyrosine with concentrations between 0 and 100 µg/ml, as indicated in the following table:

Table 8: Preparation of the tyrosine calibration curve.

Tube Number / **Reagent and Solution**	Blank	1	2	3	4	5
Concentrations of dilutions (µg/ml)	0	20	40	60	80	100
Stock solution of tyrosine (ml)	0	0,1	0,2	0,3	0.,4	0.,5
TCA (ml)	0,5	0,4	0,3	0,2	0,1	0
Na2CO3 (ml)	2,5	2,5	2,5	2,5	2,5	2,5
Shake and incubate for 10 minutes at room temperature						
Diluted Folin reagent (1/2) (ml)	0,25	0,25	0,25	0,25	0,25	0,25
Shake and incubate in the dark for 30 minutes						

The readings at 750 nm on the spectrophotometer for the different concentrations allowed for the plotting of the calibration curve**.**

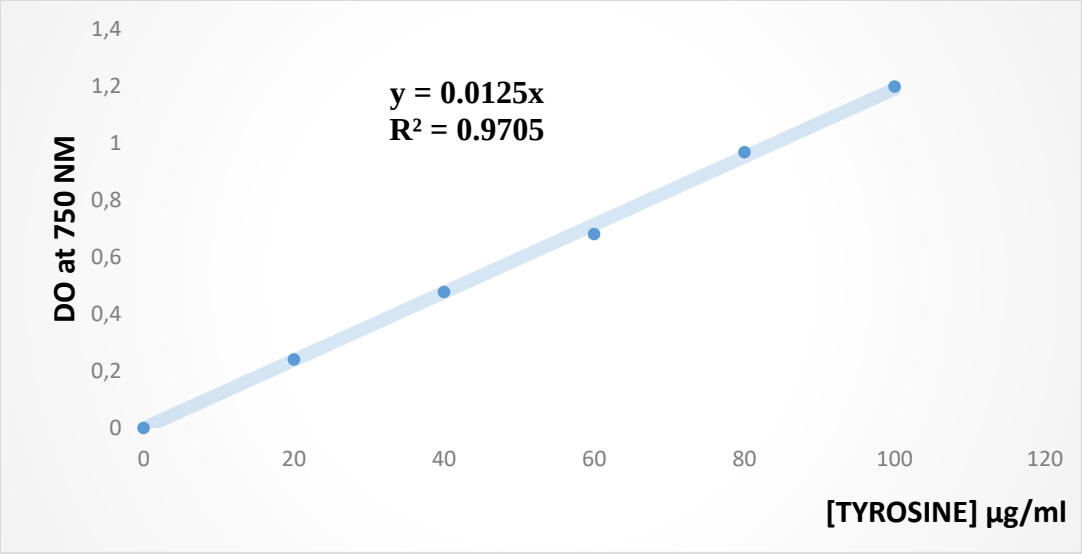

Figure 20: Tyrosine calibration curve

Appendix 3

- **Préparation des solutions tampon**

1. Tampon Citrate de Sodium (pH de 3 jusqu'à 9)

- Solution A: acide citrique C6H8O7 (0,1M).
- Solution B: le dissodique Na2HPO4 (0,2M).

Le pH est déterminé par le pH-mètre.

2. Tampon glycine (pH de 9 jusqu'à 12)

- Solution A : Glycine (0,1M) dans Nacl (0,1N).
- Solution B : NaOH (0,1N) (0,4%).

Le pH est déterminé par le pH-mètre.

Appendix 4

1. Protease Activity Assay

The proteolytic activity in enzymatic extract is determined based on the effect of protease on casein. The manipulation is conducted in two stages:

- **Enzymatic reaction**: This involves reacting the enzyme extract with the substrate (2.5% casein dissolved in sodium citrate buffer). The reaction mixture is prepared as indicated in the following table **(table9)**:

Table 9: Preparation of reaction mixture (Dakhmouche Djekrif et *al*., 2024**).**

Tube number / **Reagent and Solution**	Blank	1	2	3
Buffer volume (ml)	0,4	0,375	0,375	0,375
Enzyme extract (ml)	-	0,25	0,25	0,25
Casein solution (ml)	0,6	0,625	0,625	0,625
Shake and incubate at 40 °C in a water bath for 30 minutes				
TCA 4% (ml)	2,5	2,5	2,5	2,5
Shake and let rest for 10 minutes and the reaction mixture was filtrate				

The filtrate constitutes the solution containing the reaction product (tyrosine)

- **Measurement of activity**: The measurement is carried out as indicated in the following table **(table 10)**:

Table 10: Measurement of activity

Reagent and Solution	Blank filtrate	Filtrate 1	Filtrate 2	Filtrate 3
Na2CO3 2% in 0.1N NaOH (ml)	2,5	2,5	2,5	2,5
Filtrate volume (ml)	0,5	0,5	0,5	0,5
Diluted Folin's reagent (1/4) (ml)	0 ,25	0,25	0,25	0,25
Shake and incubate in the dark for 30 minutes Read the absorbance at 750 nm				

Each assay is performed in duplicate.

The protease activity was read at 750 nm in a spectrophotometer (UV/VIS) using a tyrosine standard curve as a reference (figure14). One unit (IU) of protease is equivalent to 1 µg of tyrosine released for 1 hour per 1 ml of enzyme solution.

Abstract

Abstract

Protease-producing yeasts were isolated from potato wastes and screened for protease production. The obtained isolates were identified as *Clavispora lusitaniae PC3* and *Rhodotorula mucilaginosa P8*. To achieve the goal of our work, we plan to investigate the optimization of protease production by these yeast strains on solid-state fermentation using tomato pomace and bread Waste mix. The strains indicated a higher enzymatic production in monoculture, 5909. 055 ± 937 UI/g for *C.lusitaniae* and and 5458. 85 ± 2027 UI/g for *R.mucilaginosa* with acidic protease being the most active with an activity of 5909. 055 ± 937 UI/g for *C.lusitaniae* and 5458. 85 ± 2027 UI/g for *R.mucilaginosa*. Further, the optimized conditions led to an enhancement of the enzymatic activity with a maximum production of 18421. 88 ± 662 U/g for *R.mucilaginosa* and 17865. 17 ± 794 U/g for *C.lusitaniae* when compared with the initial activity before the medium optimization. The increased protease activity from the optimized medium condition was approximately three times higher, which indicates that the use of PBD design was valid. The proteases displayed the highest activity at pH 4 for *C.lusitaniae* and at pH 3 for *R.mucilaginosa*. Furthermore, it was demonstrated that the protease activity was inhibited in the presence of pepstatine confirming that the produced proteases are acidic proteases.

Keywords: *Clavispora lusitaniae PC3*; *Rhodotorula mucilaginosa P8*; protease; solid-state fermentation; tomato pomace; bread Waste.

Résumé

Les levures productrices de protéases ont été isolées à partir de déchets de pommes de terre et criblées pour la production de protéases. Les isolats obtenus ont été identifiés comme étant *Clavispora lusitaniae* PC3 et *Rhodotorula mucilaginosa* P8. Pour atteindre l'objectif de notre travail, nous prévoyons d'étudier l'optimisation de la production de protéases par ces souches de levures en fermentation solide en utilisant un mélange de marc de tomates et de déchets de pain. Les souches ont montré une production enzymatique plus élevée en monoculture, avec 5909,055 ± 937 UI/g pour *C. lusitaniae* 5458,85 ± 2027 UI/g pour *R. mucilaginosa*, la protéase acide étant la plus active avec une activité de 5909,055 ± 937 UI/g pour *C. lusitaniae* et 5458,85 ± 2027 UI/g pour *R. mucilaginosa*. De plus, les conditions optimisées ont conduit à une amélioration de l'activité enzymatique avec une production maximale de 18421,88 ± 662 U/g pour *R. mucilaginosa* et 17865,17 ± 794 U/g pour *C. lusitaniae* par rapport à l'activité initiale avant l'optimisation du milieu. L'augmentation de l'activité protéase due à l'optimisation du milieu a été environ trois fois plus élevée, ce qui indique que l'utilisation de la conception PBD était valide. Les protéases ont montré une activité maximale à pH 4 pour *C. lusitaniae* et à pH 3 pour *R. mucilaginosa*. En outre, il a été démontré que l'activité protéase était inhibée en présence de pepstatine ce qui confirme que les protéases produites sont des protéases acides.

Mots-clés : *Clavispora lusitaniae* PC3 ; *Rhodotorula mucilaginosa* P8 ; protéase ; fermentation en milieu solide ; marc de tomates ; déchets de pain.

ملخص

تم عزل خمائر تنتج البروتياز من نفايات البطاطا وتم فحصها لإنتاج البروتياز. تم تحديد العوزات المحصلة على أنها *Clavispora lusitaniae PC3* و*Rhodotorula mucilaginosa P8*. لتحقيق هدف عملنا، نخطط لدراسة تحسين إنتاج البروتياز من هذه السلالات الخمائرية في التخمر الصلب باستخدام نخالة الطماطم ونفايات الخبز. أظهرت السلالات إنتاجاً إنزيمياً أعلى في وسط استزراع احادي، 5909.055 ± 937 وحدة دولية/جم لـ *C. lusitaniae* و 5458.85 ± 2027 وحدة دولية/جم لـ*R. mucilaginosa* ، مع بروتياز حمضية كانت الأكثر نشاطاً بنشاط قدره 5909.055 ± 937 وحدة دولية/جم لـ *C. lusitaniae* و 5458.85 ± 2027 وحدة دولية/جم لـ *R. mucilaginosa.*وعلاوة على ذلك، أدت الظروف المحسنة إلى تعزيز النشاط الإنزيمي مع إنتاج أقصى يبلغ 18421.88 ± 662 وحدة/جم لـ *R. mucilaginosa* و 17865.17 ± 794 وحدة/جم لـ *C. lusitaniae* مقارنة بالنشاط الأولي قبل تحسين الوسط. زاد النشاط البروتيازي من الظروف المتوسطة المحسنة بمقدار يعادل ثلاث مرات، مما يشير إلى صحة استخدام تصميم بلاكيت وبرمان. أظهرت البروتياز أعلى نشاط عند pH 4 لـ *C. lusitaniae* وعند pH 3 لـ *R. mucilaginosa*. علاوة على ذلك، تم إثبات أن نشاط البروتياز يتم تثبيطه بوجود البيبسطاتين، مما يؤكد أن البروتياز المنتجة هي بروتيازات حمضية .

الكلمات الرئيسية: *Clavispora lusitaniae PC3* ؛ *Rhodotorula mucilaginosa P8* ؛ نفايات صلب؛ تخمير ؛ بروتياز ؛ الطماطم؛ نفايات الخبز

Academic Year: 2023-2024	**Presented by :** GHITI Kaouther MOUSSA HAROUNA Chamsa

Optimization of yeast protease and characterization of the enzyme

Thesis for the Master's Degree in Applied Biochemistry

Abstract

Protease-producing yeasts were isolated from potato wastes and screened for protease production. The obtained isolates were identified as *Clavispora lusitaniae PC3* and *Rhodotorula mucilaginosa P8*. To achieve the goal of our work, we plan to investigate the optimization of protease production by these yeast strains on solid-state fermentation using tomato pomace and bread Waste mix. The strains indicated a higher enzymatic production in monoculture, 5909. 055 ± 937 UI/g for *C.lusitaniae* and 5458. 85 ± 2027 UI/g for *R.mucilaginosa* with acidic protease being the most active with an activity of 5909. 055 ± 937 UI/g for *C.lusitaniae* and 5458. 85 ± 2027 UI/g for *R.mucilaginosa*. Further, the optimized conditions led to an enhancement of the enzymatic activity with a maximum production of 18421. 88 ± 662 U/g for *R.mucilaginosa* and 17865. 17 ± 794 U/g for *C.lusitaniae* when compared with the initial activity before the medium optimization. The increased protease activity from the optimized medium condition was approximately three times higher, which indicates that the use of PBD design was valid. The proteases displayed the highest activity at pH 4 for *C.lusitaniae* and at pH 3 for *R.mucilaginosa*. Furthermore, it was demonstrated that the protease activity was inhibited in the presence of pepstatine confirming that the produced proteases are acidic proteases.

Key-words: *Clavispora lusitaniae PC3*; *Rhodotorula mucilaginosa P8*; protease; solid-state fermentation; tomato pomace; bread Waste.

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