الجمهورية الجزائرية الديمقراطية الشعبية
People's Democratic Republic of Algeria
وزارة التعليم العالي والبحث العلمي
Ministry of Higher Education and Scientific Research

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Thesis presented for obtaining the Master's Degree
Field: Natural and Life Sciences
Sector: Biological Sciences
Specialty : Biochemistry

N° d'ordre :
N° de série :

Titled

Study of the optimization of protease production in *Rhodotorula SP1* by Placket and Burman designs

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In: 13/06/2024

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Academic year: 2023 – 2024

Acknowledgements

Thanks…

*We would like to thank, first of all, **Allah** who gave us the strength, the flying and the courage to carry out this modest work so as to be able to follow our studies successfully.*

*We send our sincere thanks to our coach **Dr. Dakhmouche S** who supervised us, for her availability, her wise advice, her encouragement, her kindness, her help we are very honored to work with her.*

*Our thanks are also addressed to the jury members: **Dr. Bennamoun L and Dr. Benkahoul M** for having accepted to evaluate and judge our work.*

Finally, we offer our thanks to all those who contributed to the realization of this memory by their knowledge and advice.

Thank you!

Dedicace

Praise be to God, who illuminates the path for His servants, supports them at every step of their journey, and leads their efforts to success.

To my esteemed father, Abdelaziz, whose unwavering determination has always been an example for me to follow and an inexhaustible source of strength throughout my academic journey.

To my virtuous and beloved mother, Mokadem Rachida, who always nurtured the hope of seeing me reach this glorious day, your care and prayers have been my steadfast support.

To the love of my life and my pillar, my precious brother Omar, whose constant encouragement and unconditional support have accompanied me from the beginning until this day.

To my treasures, my dear sisters Romaissa and Ikhlas, who illuminate my life with their affection and unwavering support.

To all my friends and classmates, you have been my second family, sharing every step of this journey with me.

To my loyal partner, Naziha, for your steadfast support and valuable collaboration.

To all those who have been a support and help, family, friends, teachers, and all the educational staff, your help and encouragement have been invaluable.

Kaouther

Dedicace

My dear parents, you are for me the source of tenderness and the example of dedication that has never ceased to encourage me.

To my dear mother: Mom, your love and advice have guided me all these years. This work is above all yours. May God keep you and give you a long life so that you can enjoy even more the fruits of so many years of sacrifice.

To my dear father: Dad, I am who I am today because of you. I am so grateful to have a father like you. Thank you for everything you have done for me.

To my dear sister: Nesrine, you are my support in life.

To my brothers, Yaakoub, Fouzi, Zakaria: I love you.

To my sisters-in-law, Assma and Sabrina: Thank you for your support.

To my partner, Kaouther: Thank you for your support.

To my dear cousins: Thank you for your presence and your support.

To my colleagues: Thank you for the wonderful memories we have shared together.

To all those who know me and have encouraged me from near or far, thank you infinitely.

Naziha

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Abbreviations

ANOVA : Analyse of Variances.

FMS : Fermentation milieu solide.

FML : Fermentation milieu liquide.

H : Hour.

ml :. Milliliter

µl : Microliter.

Min :. Minute.

pH : Hydrogen potential.

Rpm :. Revolutions per minute.

TCA :. Trichloroacetic Acid.

YPGA : Yeast extract-Peptone-Gelose-Agar.

Introduction

Proteases are enzymes that catalyze the breakdown of proteins into smaller peptides and amino acids [1]. They are omnipresent; they are found in microorganisms, animals and plants. In industry, microbial proteases are the most important enzymes, accounting for approximately 60% of the total enzyme market worldwide and approximately 40% of total global enzyme sales [2] . They are commonly used in different industries including detergents [3], food industry, leather processing, meat processing, cheese making, silver recovery from photographic films , the production of digestive treatments and certain medical treatments against inflammation and healing of virulent wounds [4].

There are several classes of proteases, including serine proteases, cysteineproteases, aspartic proteases, and metalloproteases, each with specific properties and functions [5].

Aspartic proteases play a significant role in both biotechnology and medicine. Many non-Saccharomyces yeasts are known to produce extracellular proteases, but due to their significance as virulence factors, previous studies have focused on the enzymes from the Candida species. These days, increased focus is being paid for their use in industrial processes due to their low pH activities. Here are the characteristics of new acid proteases generated by yeasts that were separated from agricultural food waste [6].

Their production and activity can be influenced by various factors, such as cultivation conditions, composition of the medium and the genetic composition of the producing strain. Therefore, optimizing protease production and activity has become a key goal for researchers. The optimization process aims to achieve maximum protease activity while minimizing process costs and time.

The main objective of this work is to produce *Rhodotorula sp1* protease by an inexpensive process. Different agro-food waste is used as a production medium in order to select the best substrate and its concentration. Production is improved by the use of statistical plans. Certain characteristics of the enzyme are studied, namely: the optimal temperature, the optimum pH and the effect of pepstatin on the protease activity.

Chapter 01:

Bibliographic study

I. Yeasts

Phaff *et al.* (1968) claim that the Latin word "levure," which means "to rise," is the source of the English word yeast. Yeasts are included in the term because they can increase the foam surface area of liquid fermentation media and produce carbon dioxide during fermentation [7]. Yeasts are microscopic, unicellular fungi (figure 01).

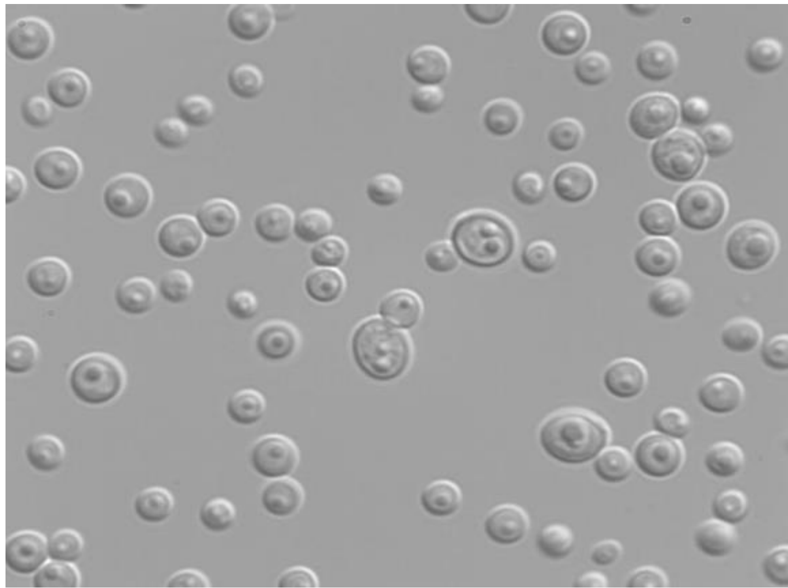


Figure.1: The yeast [8].

Cells are generally spherical, oval or cylindrical in shape; with a double-layer wall [9], the cytoplasm contains all the usual organelles of higher non-photosynthetic plants (Figure 02) [10]. Yeasts divide vegetatively by budding like *Saccharomyces* or by binary fission like *Schizosaccharomyces;* to date 1500 species of yeast have been discovered. Yeasts are chemoorganotrophic because they can use inorganic chemicals as an energy source [11]. Multiplying by budding (blastospores) and sometimes producing mycelium or *pseudomycelium.*

For millennia, people have utilized yeasts, especially in the fermentation process used to make bread and alcoholic beverages [12].

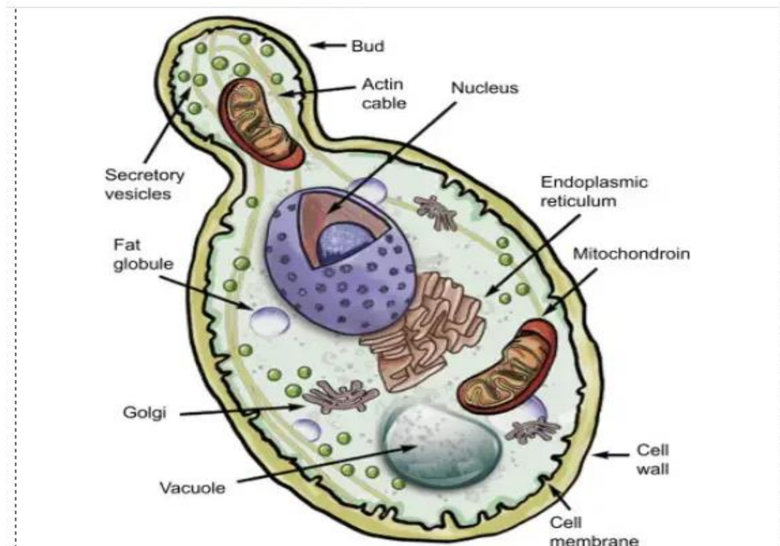


Figure.2: Presentation of a yeast cell [13].

1. Yeast reproduction

There are two types of reproduction (figure 03): sexual reproduction through sporulation, which is the union of two pre-existing cells, and asexual reproduction by budding, in which the mother cell produces two daughter cells [14].

Asexual multiplication is the predominant mode of multiplication for most yeasts. The yeast *S. cerevisiae* can also reproduce sexually and switch between these two ways of reproduction based on the environment [14].

According to Jablonowski *et al.* (2004), they bud in a favorable environment (one that contains carbohydrates and minerals) and sporulate in an unfavorable one.

Budding is the process of asexual reproduction. The mother cell initially has a little projecting mass that develops in size over time and separates when it reaches the desired size. Simultaneously, the mother cell's nucleus advances toward the edge, stretches, and some of it penetrates the bud. After then, it splits off from the mother cell, occasionally leaving a little "scar" behind [15].

This results in the production of a daughter cell, which expands until its diameter is roughly equal to two-thirds that of the mother cell before producing more buds. This population doubles every ninety minutes under ideal growth conditions, setting a record time for eukaryotic cells [16].

In an unfavorable environment (high acetate content, deficient in nutrients, high temperatures, etc.). The diploid yeast cell will sporulate, giving rise to four or eight haploid cells that will have a sluggish life. These cells are known as "ascospores" in Ascomycetes and "basidiospores"

in Basidiomycetes. The spores are released, germinate, develop, and start a fresh cycle of vegetative multiplication in either the haploid or diploid form if the environmental conditions are favorable once more [17].

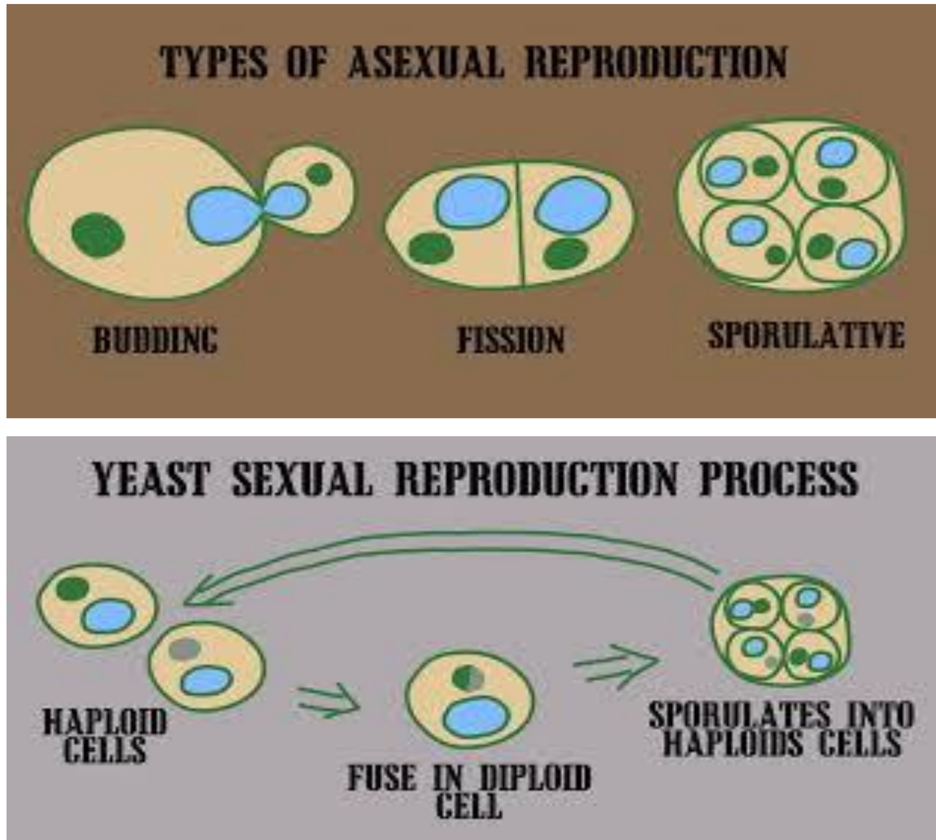


Figure .3: Yeast reproduction [18].

2. Yeast beneficial and harmful roles

Like all *fungi*, they are heterotrophic creatures, meaning that they require preexisting organic resources to grow. Humans use yeasts for commercial purposes. Among these is *Saccharomyces cerevisiae*, which is well known for its use in the food business as a fodder yeast and yeast baker in the creation of wine, beer, and spirits [12].

Yeast, a single-celled fungus, serves a dual role in both beneficial and harmful capacities. Regarding its advantages, yeast is essential to many industrial and culinary activities. It functions as a leavening agent in baking, fermenting sugars and releasing carbon dioxide to give bread a light and airy texture [19]. Furthermore, yeast is essential to the fermentation process, which produces popular alcoholic drinks like wine, spirits, and beer. Its presence improves flavor profiles and helps with preservation in the creation of several food items, including cheese, yogurt, and soy sauce. Furthermore, the potential of yeast in sustainable practices is becoming more widely

acknowledged. It plays a crucial role in the manufacture of biofuel from renewable biomass sources [20].

On the other hand, yeast can have negative consequences, especially on food preservation and human health. Some yeast species, most notably *Candida*, can cause thrush or other yeast infections that can be harmful to people's health. Uncontrolled yeast growth during food preparation can cause spoiling, which can lead to unwanted tastes, textures, and nutritional deterioration. Moreover, undesirable yeast strain contamination in commercial contexts such as winemaking and brewing can lower product quality, costing manufacturers money and alienating customers. Moreover, yeast might cause allergic reactions in certain people, which further complicates its use in food [21].

3. Biotope

Yeast, a diverse group of single-celled fungi, exhibit a remarkable adaptability to a wide range of habitats. Their primary habitat is often the surface of plants, where they thrive in environments abundant in sugars, such as the sweet sap of trees and the nectar of flowers. Here, they play essential roles in processes like fermentation, aiding in the breakdown of complex carbohydrates into simpler sugars and other byproducts. In soil, yeast are also prevalent, particularly in areas rich in organic matter, where they contribute to decomposition and nutrient cycling [20]. Additionally, yeast have been discovered in various aquatic environments, including freshwater and marine habitats, where they can survive and reproduce under specific conditions. Furthermore, yeast are known to colonize the gastrointestinal tracts of animals, including humans, where they participate in digestion and potentially influence overall health. Their ability to adapt to diverse ecological niches highlights the versatility and resilience of these microorganisms, making them fundamental players in ecosystems worldwide [22].

The following table represents each yeast and where it is found (table 01)

Table.01: Yeast habitats [18]

Habitat	Yeast Species	Isolation	References
Soil	*Cryptococcus*	Isolated from soil samples, common in decaying matter .	.[23]
Plant Surfaces	*Candida*	Found on the surfaces of plants, fruits, and flowers].	[24
Fruit juice	*Saccharomyces*	Commonly found in ripe fruits and fruit juices	[25].
Animal Tissues	*Malassezia*	Inhabits the skin and mucosal surfaces of animals .	[26]
Fermented food	*Zygosaccharomyces*	Frequently isolated from fermented food and beverages .	[1]
Marine Environments	*Debaryomyces*	Found in marine environments such as seawater and sediments .	[26]
Freshwater	*Rhodotorula*	Often isolated from freshwater sources like lakes and rivers	[1]
Insect	*Candida*	Found in the digestive tract and on the surfaces of insects.	[26]

4. Growing condition

Yeast, a diverse group of single-celled fungi, exhibit a wide array of growing conditions depending on their species and ecological niches (table 02) [27]. *Saccharomyces cerevisiae*, also known as baker's yeast, thrives optimally at temperatures ranging from 25-30°C (77-86°F) and prefers a slightly acidic pH of 4-6. It flourishes in environments rich in glucose, sucrose, and maltose, displaying a remarkable versatility in sugar utilization. This species is classified as a facultative anaerobe, capable of both aerobic and anaerobic respiration. *Candida albicans*, a pathogenic yeast commonly found in the human body, prefers warmer temperatures between 30-37°C (86-98.6°F) and tolerates a broader pH range of 4-7. It primarily consumes glucose and fructose and, like *S. cerevisiae*, is a facultative anaerobe. *Saccharomyces boulardii*, a probiotic yeast, thrives in similar temperature ranges as *S. cerevisiae* but exhibits a slightly broader optimal pH range of 4-6. It metabolizes various sugars, including glucose, galactose, and sucrose, and shares the facultative anaerobic characteristic with other Saccharomyces species. In contrast, *Schizosaccharomyces* pombe prefers temperatures between 25-30°C (77-86°F) and a pH range of 4-6. It primarily consumes glucose, sucrose, and maltose and differs from other species as an obligate aerobe, relying solely on aerobic respiration for growth. These diverse growing conditions underscore the adaptability of yeast species to a wide range of environmental factors, reflecting their importance in various industrial, medical, and ecological contexts [28].

Table 02 provides a general overview of the optimal growing conditions for these types of yeast, including temperature, pH range, preferred sugars, and oxygen requirements. Keep in mind that specific strains within each type of yeast may have slightly different preferences and requirements [29].

Table 02: Growing conditions for these types of yeast [30].

Yeast	Optimal Temperature	pH Range	Sugar Preferences	Oxygen Requirements
Saccharomyces cerevisiae (Baker's yeast)	25-30°C (77-86°F)	pH 4-6	Glucose, Sucrose, Maltose	Facultative anaerobe
Candida albicans	30-37°C (86-98.6°F)	pH 4-7	Glucose, Fructose	Facultative anaerobe
Saccharomyces boulardii	25-37°C (77-98.6°F)	pH 4-6	Glucose, Galactose, Sucrose	Facultative anaerobe
Schizosaccharomyces pombe	25-30°C (77-86°F)	pH 4-6	Glucose, Sucrose, Maltose	Obligate aerobe

Yeast, encompassing a diverse range of single-celled *fungi,* display varying nutritional requirements tailored to their ecological niches. *Saccharomyces cerevisiae*, renowned as baker's yeast, thrives in environments abundant in carbohydrates, particularly glucose, sucrose, and maltose, which serve as its primary energy sources. Alongside carbohydrates, it necessitates essential nutrients such as nitrogen, phosphorus, and a spectrum of vitamins and minerals to support robust growth and metabolism [31]. *Candida albicans*, commonly inhabiting the human body, shares a reliance on carbohydrates for energy generation, supplemented by essential nutrients vital for its proliferation within host tissues. *Saccharomyces boulardii*, a probiotic strain, exhibits a broader nutritional versatility, capable of metabolizing various sugars including glucose, galactose, and sucrose, highlighting its adaptability to diverse environmental substrates. Moreover, it necessitates essential nutrients for growth and functionality, echoing the multifaceted nutritional demands across yeast species. *Schizosaccharomyces* pombe, while sharing preferences for glucose, sucrose, and maltose, also demands specific amino acids and vitamins to support its growth and

cellular processes. These intricate nutritional requirements underscore the adaptive strategies yeast employ to thrive in varied environments, contributing significantly to industrial, medical, and scientific endeavors [32].

5. Application of yeast

Yeasts occupy a primordial place in the food industry. More than 500 species of yeast have been described, but by far the most widely used species is *Saccharomyces cerevisiae*, various strains of which are used in the preparation of bread, wine, beer and other alcoholic beverages. The global annual production of yeast is more than 2 million tonnes [33].

Yeast behaves very differently depending on whether or not it is in the presence of air.

They participate in the development of numerous food products (bread making, cheese making, brewing,) and in the production of enzymes (invertase, lactase, lipase and amylases) and in the production of biomass, glycerol as well as certain vitamins and solvents, but also to the recovery of agricultural and industrial waste and to the production of proteins. Biotechnologies and biomedical research thus widely exploit these microorganisms for the production of molecules of medical interest (e.g. production of heterologous proteins, such as the hepatitis B vaccine) [34].

Yeast has a wide range of applications across various fields:

- **Baking:** Yeast is commonly used in baking to leaven bread and other baked goods by fermenting sugars and producing carbon dioxide gas, which causes the dough to rise [33].
- **Brewing:** Yeast is essential in brewing beer, wine, and other alcoholic beverages. It converts sugars from grains or fruits into alcohol and carbon dioxide during fermentation [35].
- **Biotechnology and Biomedical Research**: Yeast, particularly *Saccharomyces cerevisiae*, is a model organism used in molecular biology and genetics research. It is valuable for studying fundamental cellular processes, gene expression, and disease mechanisms [33].
- **Biofuel Production**: Yeast can be engineered to produce biofuels such as ethanol and butanol from renewable biomass sources like agricultural residues and algae.
- **Food and Beverage Industry**: Yeast-derived enzymes and flavors are used in the food and beverage industry for various purposes, including flavor enhancement, fermentation, and food preservation [34].

- **Nutritional Supplements**: Nutritional yeast, a deactivated form of *Saccharomyces cerevisiae*, is rich in vitamins, minerals, and protein, making it a popular dietary supplement, especially among vegans and vegetarians [13].
- **Pharmaceuticals**: Yeast is used in the production of vaccines, insulin, and other pharmaceutical products through recombinant DNA technology [35].
- **Environmental Applications**: Yeast can be employed in environmental bioremediation processes to clean up pollutants and contaminants from soil, water, and air [35].

II. Protease enzymes

Proteases are the most highly sought-after hydrolases in the food and bio- industries. According to Kieliszek *et al.* (2021), they hold a leading position of approximately 65% in the global market for industrial enzyme sales, which range from 50 to 60 billion euros annually. Alkaline proteases, which are particularly useful in detergents—dominate this portion of the hydrolases that are commercially available. These proteases account for around 40% of the worldwide enzyme market because they are stable and active even at very high pH levels. They are found everywhere and are taken from plants, animals, and microbes [36].

This intricate balance between activators and inhibitors finely tunes protease enzyme activity, allowing precise control over cellular processes such as protein degradation, signaling, and maintenance of cellular homeostasis (figure4). Such regulation is essential for cellular function, ensuring proper responses to environmental cues and metabolic demands [37].

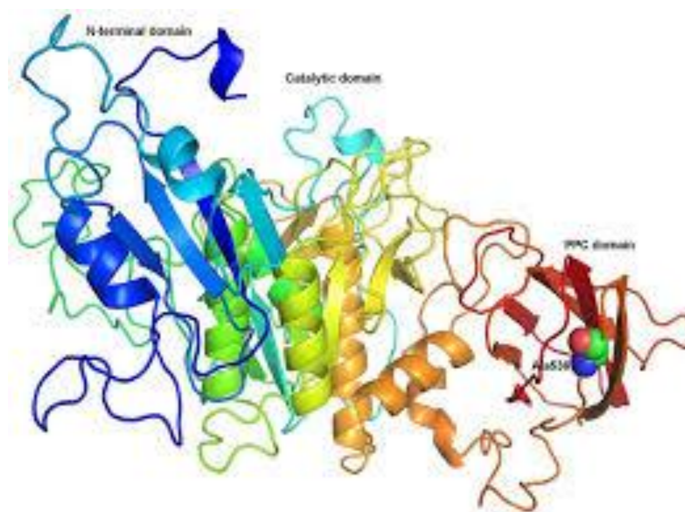


Figure.4: Structure of protease enzyme [37].

1. Definition

Proteases or proteinases belong to the class of hydrolases (EC 3.4.21-24.X). They catalyze the hydrolysis of proteins at specific sites by cleaving the peptide bond that links two amino acids into one. Proteolytic enzymes are present in all living organisms and contribute to cell growth and differentiation**.** Microorganisms have proven to be a competent and inexpensive source of protease enzymes that can produce a continuous and constant supply of the desired product. Proteases have wide application in various industrial sectors [38].

2. Origin

Proteases are derived from microorganisms, animals, and plants:

¬ **Animal proteases:** The majority of animal-derived proteases are pancreatic. Actually, they have the ability to break down the proteins in meals. Like trypsin, chymotrypsin, pepsin, and reni, they are all generated as precursors that, in certain situations, might activate autocatalytically [39].

¬ **Plant proteases**: In technology, plant-derived enzymes, particularly proteases, are arranged in declining order. Among the well-known proteases derived from plants are ficin, papain, bromelain, and keratinase [40].

¬ **Microbial proteases**: Because microbial proteases have nearly all the necessary properties for their commercial uses, they are chosen over proteases derived from other sources [41]. Numerous microorganisms, such as bacteria, actinomycetes, molds, and yeasts, are responsible for their production. They account for 40% of all enzymes on the world's market.

The great success of microbial proteases in biotechnological systems is attributed to the very broad biochemical diversity, the feasibility of mass cultivation and the ease of genetic manipulations [42].

3. Classification

Proteases are categorized as extracellular or intracellular proteases depending on where they operate. Intracellular proteases are essential for a wide range of cellular and metabolic processes. Extracellular proteases are necessary for the degradation of proteins in the external environment of the cell. More intriguingly, the latter can be extracted without requiring cell lysis, making their industrial application possible. Centrifugation is adequate to remove them from the cells [43].

Prptease wers classified in exopeptidase and endopeptidase (table 03). They within clans and families can be classified as asparagine proteases, aspartic proteases, cysteine proteases, glutamic proteases, mixed proteases, metalloproteases, threonine proteases, serine proteases, or unknown proteases based on these phylogenetic relationships and mechanisms of action [43].

Table 03: Classification of proteases [43].

Protease type	Classes and subclasses
Exopeptidases	**Aminopeptidases** -peptidyl peptidases -dipeptidyl peptidases -tripeptidyl peptidases **Carboxypeptidases** -serine carboxypeptidases -metallo-carboxypeptidases -cystiene carboxypeptidases
Endopeptidases	Serine protease Cyteine protease or thiol protease Aspartic protease or acid protease Metalloproteases

4. Proteases Characteristic

4.1. Molecular weight:

The molecular weight of aspartic proteases is generally between 30 and 45 kDa. That of metalloproteases is 60 to 65 kDa, Collagenolytic protease varies from 30 to 150 kDa and serine proteases are found between 18 to 35 kDa. According to Maquaire, cysteine proteases are a complex of soluble enzymes with molecular masses ranging from 16 to 36 kDa [44].

4.2. Temperature and thermo stability:

The temperature and thermo-stability of proteases are critical factors that influence their functionality and applicability in various industrial and biological processes. Proteases are enzymes that catalyze the hydrolysis of peptide bonds, and their activity and stability are often temperature-dependent [45].

Generally, proteases exhibit an optimal temperature range for activity, beyond which their activity may decrease due to denaturation. However, the exact optimal temperature varies depending on the type of protease and its source. For example, serine proteases, such as trypsin and chymotrypsin, typically exhibit optimal activity at temperatures around 37°C, which is close to physiological conditions in humans. These proteases are crucial for digestion and other biological processes [6].

On the other hand, proteases from extremophiles, such as thermophilic bacteria or archaea, have evolved to function optimally at high temperatures. These thermophilic proteases, such as thermolysin, are stable and active at temperatures exceeding 70°C or even higher. They are valuable in industrial processes that require high temperatures, such as protein hydrolysis for the production of biofuels or the brewing industry [45].

The thermo-stability of proteases refers to their ability to maintain their structure and activity over a range of temperatures. Proteases with higher thermo-stability are more resistant to denaturation and can with stand harsh conditions, such as high temperatures or extreme pH levels. This property is particularly desirable for industrial applications where proteases are exposed to elevated temperatures during manufacturing processes [46].

For instance, in a study by Gupta and Beg (2015), the thermo-stability of alkaline protease from *Bacillus subtilis* was investigated for its potential application in detergent formulations. The researchers found that the protease exhibited remarkable stability at temperatures up to 60°C, making it suitable for use in hot water wash cycles [47].

In another study by Siddiqui et *al*. (2017), the thermo-stability of a metalloprotease from an extremophilic archaeon was examined. The protease showed optimal activity and stability at temperatures above 70°C, highlighting its potential for applications in industries requiring high-temperature processes, such as bioremediation or biofuel production.

In summary, understanding the temperature requirements and thermo-stability of proteases is essential for their successful application in various fields, ranging from biotechnology and pharmaceuticals to food and detergent industries. Researchers continue to explore and engineer proteases with tailored temperature profiles to meet specific industrial needs [47].

4.3. pH

One of the most typical characteristics of proteases is their pH range. These enzymes are most active in three pH ranges: acidic, neutral, and alkaline. The pH of yeast protease varies depending on where it comes from [48].

The pH significantly influences the activity of protease enzymes. These enzymes catalyze the breakdown of proteins into smaller peptides and amino acids. The role of pH is paramount because it directly affects the enzyme's structure and, consequently, its function. Each protease has an optimal pH range in which it operates most efficiently. For instance, pepsin, primarily active in the acidic environment of the stomach, functions optimally at a low pH around. In contrast, trypsin, found in the alkaline environment of the small intestine, exhibits peak activity around pH 8. Deviations from these optimal pH conditions can disrupt the enzyme's active site, leading to denaturation and loss of function. Thus, maintaining the appropriate pH environment is crucial for ensuring the proper functioning of protease enzymes in biological systems [39]

4.4. Stability

The stability of proteases increases considerably when Ca^{2+} ions are added to the medium and, on the contrary, it decreases if sequestering agents are added. Thus, these proteases are inactivated in the presence of strong chelating agents (e.g. EDTA), which remove Zn^{2+}, while the removal of Ca^{2+} ions only affects their thermo-stability [37].

5. Application

The detergent industry is currently the main consumer of hydrolytic enzymes acting in alkaline environments. Particular interest has been given to enzymes for their ability to remove protein stains and thereby achieve benefits unobtainable with conventional detergent manufacturing technologies. The main use of compatible proteases in detergents concerns laundry detergent formulations, and also dishwashing formulations, where they are widely used Tannery The chemical technique for removing hair from animal skins produces a very polluted effluent in addition to being expensive and drastic. The alternative to this process is the use of proteolytic enzymes[49].

This advantage is preferentially possible if the protease is active in an alkaline medium .

¬**Medical uses** Collagenases with protease activity are increasingly used in therapy in drug formulations. A novel semi-alkaline protease with significant collagenolytic activity was produced by *Aspergillus niger* LCF9 [39].

¬**Food industry** Microbial proteases have been widely exploited in the food industry.

These enzymes have been used in the preparation of protein hydrolysates with high nutritional value. Protein hydrolysates produced from casein, whey and soy play an important role in the

regulation of blood pressure and are used in the formulation of hypoallergenic children's foods, in dietetic products and for the enrichment of juices [42].

¬**Waste treatment** Alkaline proteases find many applications in the field of domestic waste management and the food industry. Fibrous proteins from hair, nails and even feathers are very abundant wastein nature [39].

These can be transformed into biomass, protein concentrates or amino acids using the action of proteases of certain microorganisms [49].

III. Production of protease

1. Costs

In industry, it is important that the protease production process is low cost. Knowing that the substrate represents 40% of the cost of enzyme production [50].

In this case, it will be interesting to product protease from agro-food and industrial waste, raw material costs can be relatively low, as these wastes are often available at low cost or even free of charge. This can significantly reduce production costs compared to using food-grade raw materials. Protease production can be carried out by liquid fermentation or solid fermentation [50]

2. Fermentation solid

Solid fermentation involves the use of solid substrates, such as agricultural crop residues or industrial by-products, in which microorganisms produce enzymes while colonizing the surface of solid particles. This method may offer additional economic benefits because it uses waste materials that are often available at low or no cost. Additionally, solid-state fermentation can be performed on a larger scale, potentially reducing production costs per unit of enzyme produced [51].

3. Fermentation liquid

In the liquid fermentation process, waste is transformed into a liquid substrate, promoting microbial growth and enzyme production. This method has economic advantages, as it requires less expensive equipment and relatively simple fermentation conditions. In addition, it allows for better regulation of fermentation parameters, such as temperature, pH, and nutrient concentration, which can improve the efficiency of enzyme production [52].

Fermentation liquids offer numerous advantages in various culinary and health applications. Firstly, they serve as a natural preservative, prolonging the shelf life of foods by creating an environment that inhibits the growth of harmful bacteria. This preservation method has been utilized for centuries, allowing communities to store perishable items for extended periods without the need for refrigeration or artificial additives [51].

Furthermore, fermentation liquids contribute to the development of complex and rich flavors in fermented foods. The liquid acts as a medium for the growth of beneficial bacteria and yeast, which break down carbohydrates and proteins into flavorful compounds such as acids, alcohols, and aromatic compounds. This results in uniquely tangy, savory, or sour taste profiles that enhance the culinary experience [52].

Moreover, fermentation liquids are valuable sources of probiotics, beneficial microorganisms that promote gut health. Consuming foods or beverages fermented with these liquids can support digestion, improve nutrient absorption, and strengthen the immune system. Incorporating fermented foods into one's diet has been linked to various health benefits, including better digestion, reduced inflammation, and enhanced overall well-being [52]

Additionally, fermentation liquids can be utilized as starter cultures for future fermentations, providing a natural and cost-effective method for initiating the fermentation process. By inoculating new batches of food with a small amount of fermented liquid, individuals can ensure consistency in flavor and quality while reducing reliance on commercial starters or additives.

In summary, fermentation liquids offer a multitude of advantages, including preservation, flavor enhancement, probiotic support, and sustainability in food production. Incorporating fermented foods and beverages into one's diet can contribute to a healthier life style and a deeper appreciation for the art of fermentation [53].

4. Factors influencing proteolytic production

4.1. Substrat

The substrate for proteases is typically a protein or peptide molecule. Proteases catalyze the hydrolysis of peptide bonds within proteins, breaking them down into smaller peptides or individual amino acids. Each protease enzyme has its own specific substrate preferences, recognizing and cleaving particular peptide bonds based on the sequence and structure of the protein or peptide substrate [54] **.**

4.2. pH

The optimum pH varies depending on the specific protease and can range from acidic to alkaline. Maintaining the optimum pH promotes maximum enzyme activity while ensuring the stability of the producing microorganisms [36].

4.3. Production temperature

Temperature was also a critical factor. It affects the growth of microorganisms and the production of enzymes. A suitable temperature, usually in the range of 25°C to 40°C, is necessary to promote optimal growth of microorganisms while preserving the stability of the produced enzyme [15]

4.4. Thermo stability

Proteolytic enzymes produced by microorganisms vary in their ability to withstand high temperatures. In industrial applications, thermostable proteases are often desirable as they can remain active at elevated temperatures, which can increase reaction rates and reduce the risk of contamination. Microorganisms capable of producing thermostable proteases are valuable for processes requiring high-temperature conditions, such as laundry detergents, food processing, and biofuel production [47].

4.5. Activators

Activators can be added to the production medium to boost enzyme activity. These substances, such as metal ions or certain organic compounds, can improve the efficiency of proteases by increasing their catalytic activity [55].

4.6. Production time

Is also a crucial factor to consider. It usually takes some time for microorganisms to produce and secrete enough enzymes into the production medium. This time may vary depending on the microbial strain used, the culture conditions, and the specific characteristics of the production process.

4.7. Agitation

Agitation plays a crucial role in microbial cultures by ensuring proper mixing of nutrients and oxygen, facilitating the growth of microorganisms, and improving mass transfer. Adequate agitation can enhance proteolytic enzyme production by optimizing nutrient availability, maintaining uniform conditions throughout the culture medium, and preventing the accumulation of toxic metabolites. Additionally, agitation can induce shear stress on microbial cells, which may trigger the production of proteases as a defense mechanism or response to environmental cues [56].

4.8. Nitrogen sources

Are essential for the growth of enzyme-producing microorganisms. Proteins and peptides are often used as nitrogen sources in production media to stimulate the growth of microorganisms and the production of proteolytic enzymes. The choice of nitrogen sources and their concentration

must be carefully balanced to optimize enzyme production while minimizing costs and avoiding adverse effects on fermentation [56].

5. Optimization

The optimization process is of great importance for the industrial production of enzymes and especially in the case of overproduction, manipulation of the environments represents the best alternative to genetic manipulation [57].

Different types of experimental designs are used in biology and particularly in the field of biotechnologies, they allow the optimization of the production of microbial enzymes. These methods make it possible to vary several factors at the same time, in order to study their effects on the parameters to be studied [57]. The objective of experimental designs is to best choose the experiments to be carried out in order to discover the rules of evolution of a quantity of interest according to operating variables. These rules are most often translated by a mathematical formula or by instructions for use [56].

The formulas found are essentially practical, that is to say they give an approximate mathematical representation of the phenomenon in a limited region of the experimental space [58].

5.1. OFAT (One Factor at a Time)

OFAT is a problem-solving technique that identifies the critical causes of an effect from a set of potential causes. The approach adopted is to modify a single factor, and keep everything else constant (all other factors). Hypothesis testing is the most commonly used method for OFAT

This technique is time-consuming and expensive. In addition, when a large number of variables are considered, it does not have the ability to detect the true optimum and does not take into account interactions between factors [59].

5.2. Plackett and Burman (1946) design

Plackett and Burman published their study in 1946 in the journal Biometrika.

They explain how to construct designs to study a large number of factors in a few trials. They assign two levels to each factor and assume that all interactions between factors are negligible compared to the main effects. This plan is presented in the form of orthogonal square matrices containing only +1 and -1. These are Hadamard matrices. They indicate all the matrices (N, N) from N = 8 to N = 100 that can be constructed. He had not yet found the construction of the matrix (96, 96) at the time of their publication [58].

These plans are widely used in improving enzyme production by different microorganisms because of their effectiveness.

The method of Plackett and Burman (1946) has been widely used in various industrial fields. These designs are previousely used for selection of factors influencing the production of asparaginase production by a novel isolated bacterium *Brevibacillus borstelensis ML12* [60], Protease from *Clavispora lusitaniae* [10], β-galactosidase from *Talaromyces pinophilus* [61] et l'α-amylase de *Bacillus amyloliquefaciens KCP2* [62].

5.3. Box and Wilson design

These experimental designs (Centered Composite Designs) coupled with the response surface is the most widely used. Centered composite designs include a factorial design or a fractional factorial design that allows the study of factors at 05 levels -α ,-1, 0 +1, + α for Box - Wilson design and at 03 levels -1, 0, +1 for Box-Benhken design.

These experimental designs coupled with RSM represent a set of experimental strategies, mathematical methods and statistical inferences to construct and explore an approximate functional relationship between a response variable and a set of design variables. RSM is only useful for a small number of variables (up to five), due to the high number of experimental runs required. It is used to explain the combined effects of all factors in a fermentation process [63].

This approach was used to study the cumulative interactive effect of macronutrients in the media, and to optimize their concentration in order to improve the production of protease from:

Several studies utilized the Box-Wilson method for optimizing enzyme processes. One such study conducted by Smith *et al.* (2019) focused on enhancing the activity of a specific enzyme crucial for biofuel production. Through the application of the Box-Wilson method, they systematically varied parameters such as temperature, pH, and substrate concentration to identify the optimal conditions for maximum enzyme efficiency.

In another investigation by Johnson and colleagues (2021), the Box-Wilson method was employed to optimize the production of a therapeutic enzyme used in treating genetic disorders. By carefully designing experiments and analyzing the response surface, they were able to pinpoint the precise combination of factors that yielded the highest enzyme activity.

Additionally, a study by Lee *et al.* (2022) applied the Box-Wilson method to enhance the catalytic performance of an enzyme used in industrial processes. Their research demonstrated how systematic optimization using this method led to significant improvements in enzyme stability and reaction kinetics, ultimately increasing the overall efficiency of the enzymatic process [64].

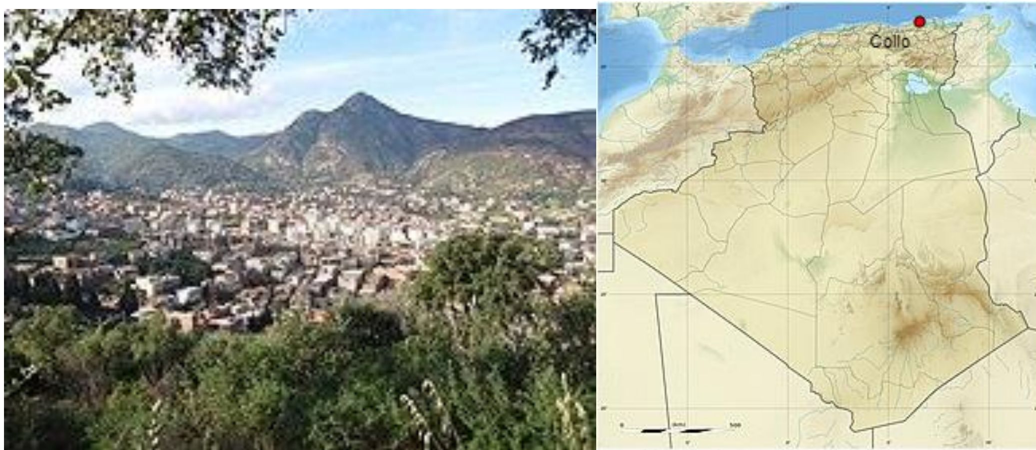
Materials and methods

The work was carried out at the Microbiological Engineering and applications Laboratory (MEA). it focuses on the study of protease production by a new strain of the *yeast Rhodotorula Sp* 1 isolated from honey, then the production was improved by using the Plackett-Burman designs and finally, the study of its characteristics was realised, namely the effect of temperature and pH on proteolytic activity.

1. Microorganism

1.1. Sampling and Yeast Strains

The new proteolytic strain is isolated from honey coming from the Collo mountains (wilaya of Skikda) located in the Collo massif region (figure a) in northern Constantine, in the North-East of Algeria (figure b). The climate is Mediterranean, mild in winter and warmer in summer. There are more than 3,000 hours of sunshine per year and the average temperature is 17.9°C and the average annual precipitation is around 1,000 mm.



a: Collo massif **b :Collo Location**

Figure.5 : Collo, Wilaya of Skikda

1.2. Yeast isolation

Isolation is carried out using honey figure 06 with a pH of 3.6 brought back into a tightly closed glass jar. To prepare a stock solution, 10 g of honey, and Tween 80 at 0.45% added to 90 ml of sterile distilled water. shake well.

Figure.6 : Honey

1.3. Dilutions Preparation

- After homogenization, prepare a series of decimal dilutions of stock solution up to 10^{-7} [65].

-Pour the sterile YGC medium into petri dishes

-After solidification of the medium, 100 µl of each dilution are spread out separately on the surface of the medium with a rake [66].

-Incubate at 20°C for 2-7 days

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

1.4. Preparation of mediums

a/ YPGA medium

Dissolve in 1l of distilled water:

- 10g of Peptone (meat extract)
- 5g of Yeast extract
- 20g of Glucose
- 20g of Agar

b/MA medium

Dissolve in 1l of distilled water:

- 5g of Peptone
- 3g of Yeast extract

-10g of Glucose

- 20g of Agar

- 3g of Malt extract

- 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.

1.5. Yeast replicate and purification

- After macroscopic observation, the purification of yeast was carried out by the streak method on YPGA. The incubation of the strains is realized at 30°C for 2 to 3 days [67].

1.6. 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.

1.7. Phenotypic identification of strains:

The phenotypic identification of isolates is based on morphological and biochemical criteria.

1.8. Morphological characteristics:

Morphological characteristics of purified yeast isolate was performed in YPGA at 30°C for 3 days. After incubation, cell shape and vegetative reproduction form were studied via microscopic examination. The appearance of yeasts on solid medium was examined based on their cultural characteristics such as colony colour, texture, margin, and the surface.

1.9. Biochemical identification

Identification of the yeast strain (Assimilation of carbon compounds: API ID32C Gallery)

The ID 32C gallery is a standardized system for yeast identification, containing 32 miniaturized assimilation tests. This manipulation was carried out within the Microbiological and Application Engineering Laboratory.

Principle of the API gallery

Gallery 32C has 32 cupules each containing a carbonaceous substrate in dehydrated form (Table 04), filled with a semi-solid medium (API C Medium) (Table 04) (the medium can be replaced by a tube containing 2 ml of distilled water without additives or YPG medium) previously inoculated.

A few colonies are transferred into sterile distilled water until Mac Farland 2 opacity. 250 µl of this suspension are transferred into an ampoule of semi-solid API C medium provided by the manufacturer. After homogenization, each well in the gallery is inoculated with 135 µl of seeded medium. The gallery covered with a lid is incubated at 30°C for 48 hours (figure 07).

Visual readings are taken after 24 and 48 hours of incubation. The possible presence of a cloudiness is noted after comparison with the control (O) (position 1.F on the gallery). A cupule that is cloudier than the control indicates a positive reaction and fermentation of the compound.

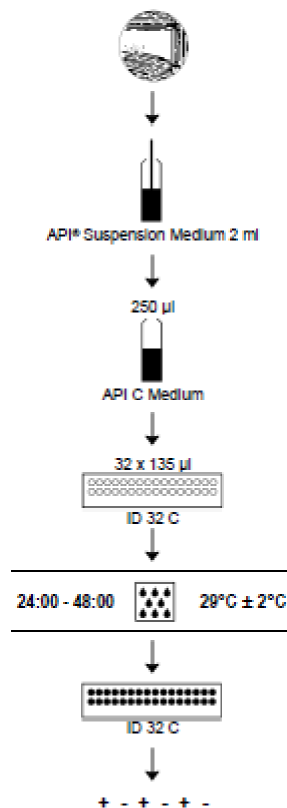


Figure.7 : Yeast identification procedure using the ID 32C gallery

Table 4: The composition of the ID 32 C band

38 Cupules	Tests	Substrate	mg/cupule
1.0	GAL	D-GALactose	0.70
1.1	ACT	cycloheximide (ACTidione)	0.014
1.2	SAC	D-SACcharose (sucrose)	0.66
1.3	NAG	N-Acetyl-Glucosamine	0.64
1.4	LAT	LacTic acid	0.64
1.5	ARA	L-ARAbinose	0.70
1.6	CEL	D-CELlobiose	0.66
1.7	RAF	D-RAFfinose	2.34
1.8	MAL	D-MALtose	0.70
1.9	TRE	D-TREhalose	0.66
1.A	2KG	potassium 2-KetoGluconate	1.09
1.B	MDG	Methyl-αD-Glucopyranoside	1.92
1.C	MAN	D-MANnitol	0.68
1.D	LAC	D-LACtose (bovine origin)	0.70
1.E	INO	INOsitol	0.70
1.F	0	No substrate	-
0.0	SOR		2.72
0.1	XYL		0.70
0.2	RIB		0.70
0.3	GLY		0.82
0.4	RHA		0.68
0.5	PLE		0.66
0.6	ERY		1.44
0.7	MEL		0.66
0.8	GRT		0.76
0.9	MLZ		0.66

2. Demonstration 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 [68].
- Inoculate the media with yeast and incubate at 30°C for 4 to 5 days.

- 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[68].

3. Inoculum Preparation

To prepare the inoculum, 50 ml of YPGA medium is poured into 250 ml Erlenmeyer flasks and then inoculated with *Rhodotorula Sp 1* 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.

3.1. Cell Counting

The number of cells in the yeast suspension is estimated through direct counting using a Thomas cell (0.1mm, 1/400mm²) at a magnification of x40 (figure 08). A drop of the yeast suspension is placed between the cell and the coverslip using a pasteur pipette, and yeast cells are then counted within the grid This counting method enables the determination of the total number of cells per unit volume of culture medium.

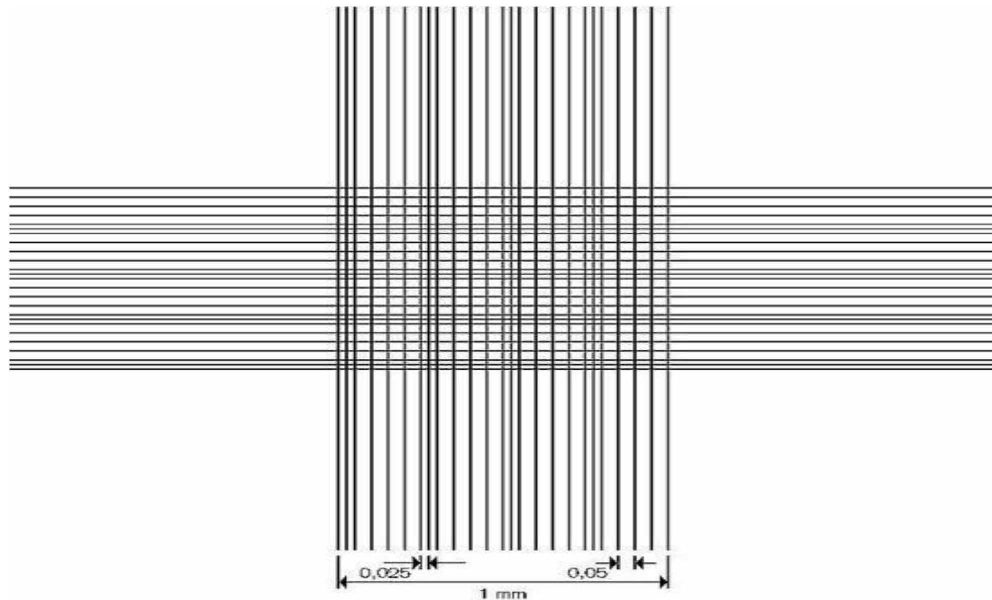


Figure.8: Thoma counting cell. (https://www.novolab.eu/cellule-de-numeration-thoma-avec-pinces.html)

4.Protease Production

4.1. Substrate preparation

Several agro-food wastes were studied for the production of *Rhodotorula sp1* protease in order to select the best one for the synthesis of the enzyme. These agro-food wastes used as substrate are tomato pomace (TP), waste dates (WD), whey (W), wheat bran (WB), orange peel (OP) and the preparation and composition of these substrates are as follows:

4.1.1. Tomato pomace

It was provided from the Ben Azzouz factory, Annaba. The tomato pomace (TP) is exposed to open air at a temperature of 25 to 30°C for a period of 3 to 4 days. Afterwards, it is ground using a domestic electric mill before being sifted to obtain a substrate in the form of flour.The waste is kept in tightly closed boxes. Tomato pomace is rich in carbohydrates, proteins and minerals (table 05)

Table.5: Chemical composition, minerals content and fatty acids of tomato peel [69].

Chemical composition (g/100 g)		Minerals content (mg/100 g)	
Protein	10.50	Ca	160
Fat	4.04	Mg	149
Ash	5.90	Fe	1.50
Carbohydrates	78.56	Cu	1.10
Fatty acids (%)		Cr	0.06
Myristic acid	0.34	Co	0.01
Palmitic acid	15.19	Ni	0.66
Stearic acid	6.84	Zn	3.12
Total Saturated	22.37	Mn	1.40
Palmitoleic acid	1.82	Na	73.6
Oleic acid	19.14	Se	0.01
Linoleic acid	52.41	Pb	0.05
Linolenic acid	4.26	Cd	0.02
Total Unsaturated	77.63	K	1097

4.1.2. Orange waste

Peel orange waste was provided from UNAJUC firm Ramdhan Djamel, Skikda. Algeria. The wastes are dried in the open air, and then, grounded and sifted and preserved in hermetically sealed boxes. The chemical composition of orange waste was presented in table 6.

Table.6: Chemical composition of orange waste

Composition	Moyenne	Variation
Proteins	6.99	5.59-9.09
Fat	1.39	0.69-2.59
Carbohydrate	57.69	-
Organic acid	7.9	-
Salt minerals	3.35	2.65-3.98

4.1.3. Wheat bran

Wheat bran was bought from the market. It was grinded to achieve a uniform particle size and was sieved the ground bran to obtain a fine and homogeneous powder. The flour is kept in well-closed box. The chemical analysis of wheat bran was carried out and the results are presented in the(table7).

Table.7: Chemical composition of wheat bran

Chemical composition	Rate %
Ashes	3.10%
Humidity	10.95%
Fat	4.12%
Proteins	16%
Reducing sugar	2.3%
pH	6.40

4.1.4. Whey

It was provided by Numidia Dairy Chaab Erssas Industrial Zone, Constantine, transported in tightly closed containers. Its pH was pH 4.46, from cheese manufacturing. It is stored at 4°C for a short period of 24-48 hours or at -20°C for a longer period.

- Whey traitment

The whey is filtered through cheesecloth to remove impurities. Before its use, the whey undergoes a thermocoagulation treatment under the combined action of pH (adjusted to 4.6) and temperature at 100°C for 30 min in order to precipitate the caseins which will then be eliminated by centrifugation at 4000 g for 15 min (or by filtration). The supernatant or filtrate constitutes the basic medium and is used to prepare the culture medium [70].

Table.8: Chemical composition of whey

Chemical composition	Rate %
Water	93.5%
Salt mineral	0.9%
Proteins	0.8%
Fat	0.6%
pH	4.6

4.1.5. Dates

Origin: We brought it from the Tolga region, Wilaya of Biskra

The wastes are dried in the open air, and then, grounded and sifted and preserved in hermetically sealed boxes. Chemical composition of dates waste was determined (table 09).

Table.9: Chemical composition of dates waste

Chemical composition	Rate
Humidity	10%
Salt mineral	4.5%
Proteins	3.22%
Sugar	90%
pH	4

NB: All The chemical composition of all these wastes were previously measured in the laboratory.

4.2. Fermentation

Liquid fermentation is carried out in 250 ml Erlenmeyer flasks containing 2% waste substrates. Each Erlenmeyer flask must be thoroughly mixed. The flasks are then stoppered with cotton wool, covered with aluminum foil, and sterilized in an autoclave at 121°C for 20 min.

After cooling, the media were inoculated with 10^7 cells/ml. The Erlenmeyer flasks are then placed in a shaker incubator at 30°C for 48h at 150r pm. All experiments are performed in duplicate.

4.2.1. Separation des enzymes proteolytique

After incubation for 48 hours, the fermentation media are centrifuged at 10000 g at 4°C for 10 min. The supernatant was filtered if necessary using Whatman paper n°1 and the filtrate was used as a crude enzyme.

4.2.2. Proteolytic activity assay

The proteolytic activity in enzymatic extract of *Rhodotorula sp1* was measured according to method described by Benkahoul *et al.,* (2020) with minor modifications using casein as substrate [71]. 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 Na_2CO_3 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 (figure 09). One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmole tyrosine/min.

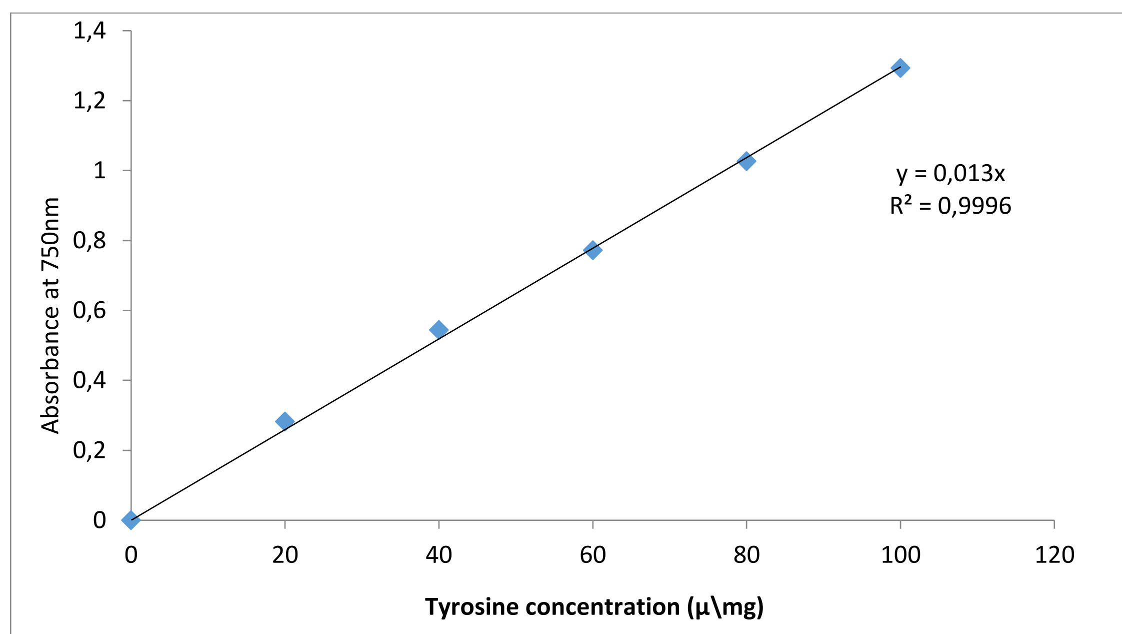


Figure.9 : Tyrosine calibration

5. Optimization method

The optimization of the production of *Rhodotorula sp 1* protease is studied to improve it. For this we used the statistical designs of Plackett and Burman (1946).

5.1.Plachett and Burman s experimental design

It was used in our study for the improvement of the production of protease by *Rhodotorula sp1*.In this study,we tested the effect of seven factors (glucose,sucrose,yeast extract,corn steep-liquor,tween -20,$NaNO_3$, $MgSO_4$) each factor at tow concentration levels as shown in the (Table 10).

Table.10: Concentration of factors

Factors	Level		Units
	-	+	
Glucose	0	1	%
Sucrose	0	1	%
Error	-	-	-
Yeast extract	0	2	%
Corn steep-liquor	0	2	%
Error	-	-	-
Tween-20	0	1	%
$NaNO_3$	0	0.3	%
Error	-	-	-
$MgSO_4$	0	0.1	%
Error	-	-	-

Table.11: Marix generators for N=8,12,16,and 20 trials Plachett and Burman (1946).

N=8 + + + - + - -
N=12 + + - + + + - - - + -
N=16 + + + + - + - + + - - + - - -
N=20 + + + + + - + - + + - - + + - - + - + - - - -

Higher level +, Lower level –

Table.12: The plachett and burman matrix for the study of 11 variables and 12 experiments

Experiences	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, K are error variables : + (higher level) ; - (lower level).

Table.13: the different factors studied and their corresponding concentration.

Essai	Glucose	Sucrose	Error	Yeast extract	Corn Steep-liquor	Error	Tween-20	$NaNO_3$	Error	$MgSO_4$	Error
1	0.5g	0.5g	-	1g	1000µl	-	0	0	-	0.05g	-
2	0	0.5g	-	0	1000µl	-	500µl	0	-	0	-
3	0.5g	0	-	1g	0	-	500µl	0.15g	-	0	-
4	0	0.5g	-	1g	1000µl	-	500µl	0.15g	-	0	-
5	0	0	-	0	1000µl	-	0	0.15g	-	0.05g	-
6	0	0	-	1g	0	-	500µl	0	-	0.05g	-
7	0.5g	0	-	0	1000µl	-	500µl	0.15g	-	0.05g	-
8	0.5g	0.5g	-	0	0	-	0	0.15g	-	0	-
9	0.5g	0.5g	-	0	0	-	500µl	0	-	0.05g	-
10	0	0.5g	-	1g	0	-	0	0.15g	-	0.05g	-
11	0.5g	0	-	1g	1000µl	-	0	0	-	0	-
12	0	0	-	0	0	-	0	0	-	0	-

The results are processed by the software.

6. Characterization method

6.1. Determination of the optimal temperature

The effect of temperature was studied by measuring the enzymatic activity at different temperatures varying from 30°C to 80°C in pH=4

6.2. Determination of the optimum Ph

The influence of Ph on the enzymatic activity is determined by measuring the activity at different Ph from Ph 3 to Ph7 at 40°C.

6.3. Effects of Pepstatin on Enzyme Activity

The effect of pepstatin at 1 µg/ml on protease activity was studied After incubating at 60°C for 10 min, the activity is measured, and compared with that of the control incubated, under the

same conditions without effectors. All assays were repeated three times, and the values presented are averages of the three experiments.

Results and Discussion

This work consists of the production of yeast protease. We will present, firstly, the results of the results of the macroscopic and microscopic observation of the strain, its identification and also its capacity to produce the protease qualitatively and quantitatively. Secondly, we present the results of the selection of the type of yeast protease studied. In addition, we show the results of the optimization of its production and finally those of the study of some biochemical characteristics such as pH and optimal temperature.

1. Yeast study

The purified yeast isolated from honey is studied with a code of H8. Macroscopic and microscopic characters, filamentation and sporulation tests and biochemical characters are studied and the results are demonstrated in table 14.

Table.14: Identification based on biochemical (ID 32C) and morphological characters

Origin	**Code**	**Macroscopic characters**	**Microscopic Characters**	**Filamentation on R.A.T.**	**Sporulation**	**Biochemical characters (ID 32C)**
Honey	H8	Colonies pink, smooth, shiny Whole contour (regular)	Cells ovoid to spherical, 3-6x2-10 µm in size Multilateral budding and fissiparity Isolated or in pairs	-	-	5461 7401 17

According to morphological characters (Figures A and B) and the identification of the yeast carring out by Galleries 32 C, the result gave the code of 5461 7401 17 and interpreted using the APIWEB download provided by the manufacturer. The strain was found to belong to the genus *Rhodotorula* .

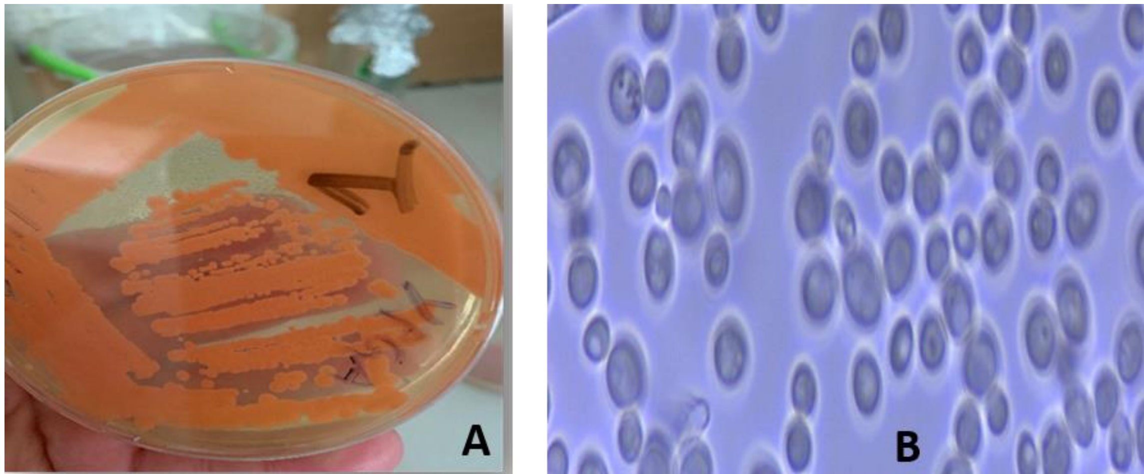


Figure.10: *Rhodotorula sp1*

A : Culture on YPGA at 30° for 48h

B: Microscopic appearance (x100)

The results of the demonstration of proteolytic production show the presence of a clear ring around the colony. This reflects the decomposition of casein by *Rhodotorula Sp1* protease (figure 11).

Figure.11: Proteolytic lysis zone in *Rhodotorula sp1*

2. Production of protease

2.1. Selection of the best waste and the best pH

Different agro-food wastes were studied for the production of protease in *Rhodotorula Sp1*. These wastes include whey, wheat bran, tomatoes, oranges, and dates. The production was carried out in liquid fermentation, and protease activity was measured at different pH: pH 4, pH 7, and pH 9. The results are presented in the figure 12.

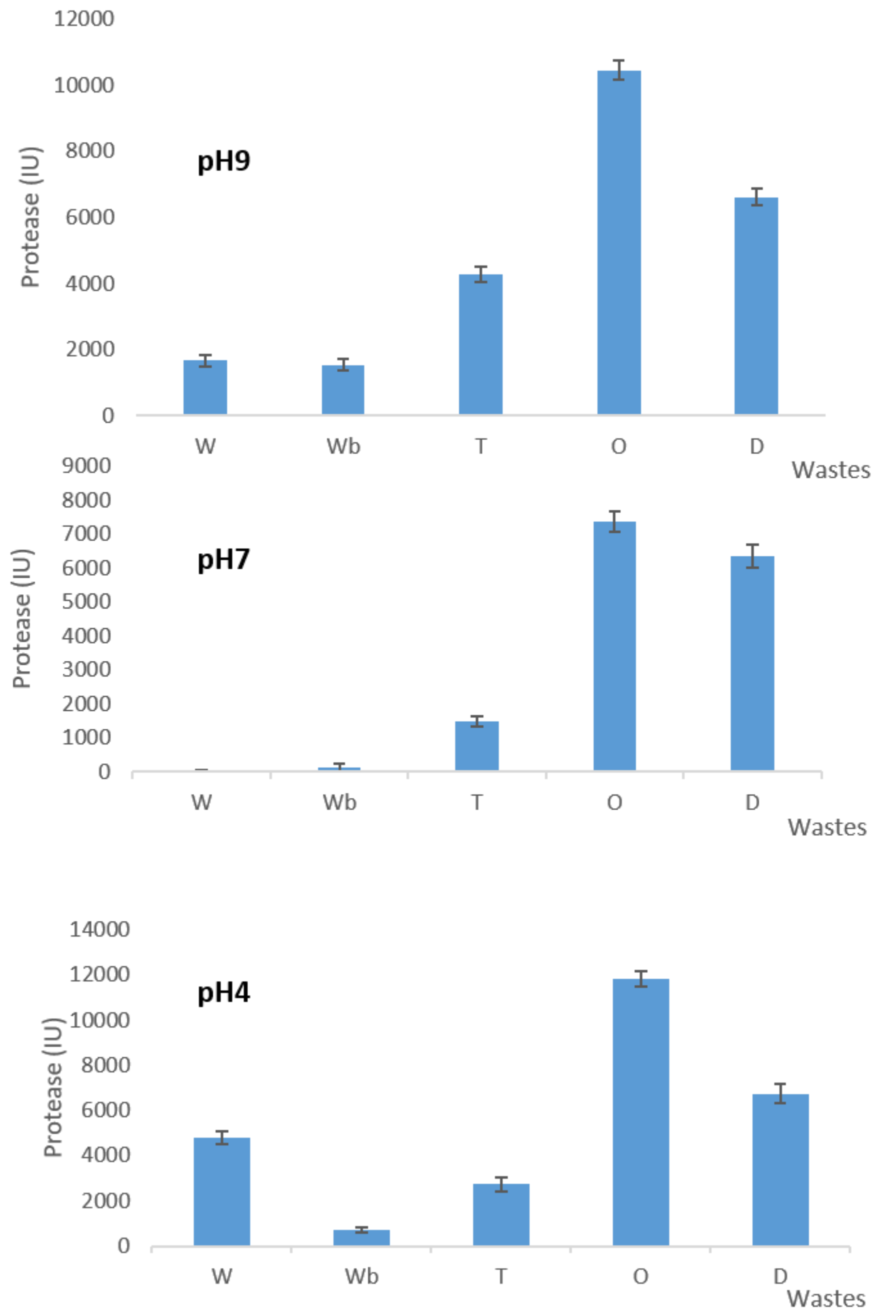


Figure.12: Effect of different wastes on protease production from *Rodotorula sp1*

According the results in the figure, it appears that the *Rhodotorula sp1* yeast is capable of growing on different agri-food waste.

However, the activity at pH 4 is better in the presence of orange waste (peel) (11815 IU), followed by date waste (6738 IU) and whey (4800 IU).

At pH7, orange waste is the best substrate for protease production (7353 IU) and an activity of 6338 IU was obtained with date waste. Less significant activities were observed with tomato waste, whey and wheat bran.

At pH9, orange waste also seems to be the best medium for protease synthesis (7353 IU), date and tomato waste gave a production of 6615 IU and 4270 IU respectively.

Orange and date waste constitute the best substrates for the production of *Rhodotorula sp1* protease, followed by tomato waste. However, wheat bran and whey do not promote enzyme production.

This can be explained by the difference in the biochemical composition of the waste (tables) which leads to the difference in the C/N ratio of the media. Probably the C/N ratio of the orange waste medium is the best followed by that of the date waste.

Orange waste has already been the subject of several studies on the production of enzymes and it seems to constitute a good substrate for the growth of microorganisms and the production of metabolites: alpha amylase from *Aspergillus niger, Aspergillus oryzae and Penicillium sp* [72] [73].

.Our results corroborate those of Benkahoul *et al.* 2017, who observed that the yeast *Aspergillus oryzae* can produce proteases from orange waste [71].

Sadoun (2023) demonstrated that orange peels contain antioxidants such as vitamin C, which protect enzymes from oxidation. This supports our observations of high enzymatic activity in orange waste.

(Mechakra-Maza *et al.* 2002) confirmed our results, stating that *Aspergillus niger* showed good results in an acidic medium (pH=5) using orange waste).

We conclude that *Rhodotorula sp1* produces acid, neutral, and alkaline proteases more effectively using waste from oranges and dates. However, the highest production occurs with acid protease (pH 4) when using orange waste.

2.2. Selection of best substrate concentration for protease production

The effect of waste orange concentrations ranging for 1% to 30% on *Rhodotorula sp1* protease synthesis is depicted in (Figure 13).

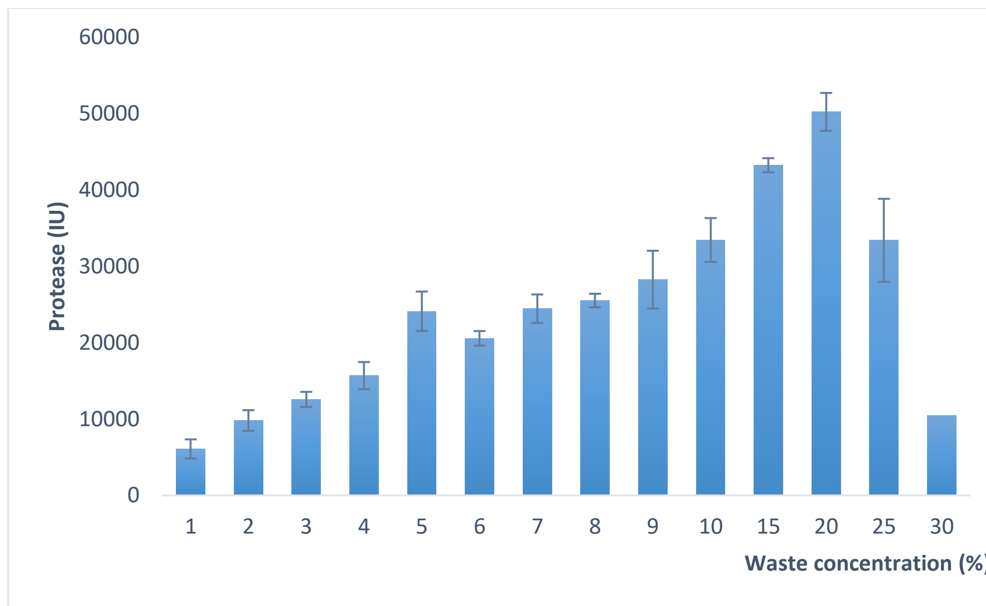


Figure.13: The effect of waste orange concentrations ranging for 1% to 30% on *Rhodotorula sp1*

Protease production increases progressively for 1% to 20% of orange waste. Beyond this concentration, production decreases. This concentration will be maintained for the remainder of our work.

At this concentration, the C/N ratio is probably optimal for yeast growth and protease production.

_ Orange pulp waste has been the subject of various studies on the alpha-amylase of *Aspergillus niger*. A concentration of 4% was found to be the best for enzyme production (Dakhmouche.S., 2001). The same concentration was also the best for protease production by *Aspergillus oryzae Ahlburg* (M. Benkahoul et al., 2017). For the production of alpha-amylase by *Penicillium camemberti PL21*, a concentration of 1% resulted in the highest enzymatic production (B.Leila et al., 2010).

The difference in this concentration is due to the difference in the type of orange waste used.

Previous studies used orange pulp, while in our work we used orange peels. SO, the biochemical composition is different (see table 06) and certainly the C/N ratio too.

_Orange peels were the subject of a study on the production of three extracellular enzymes as a source of carbon, namely polygalacturonase, cellulase and aspartic protease from Botrytis cinerea Pers, and the results were positive (Z. Karmona et al., 1990).

2.3. Optimization of Protease Production

To optimize the production of protease from *Rhodotorula sp1*, we improved the production medium by studying various factors to determine their effect on enzyme production. For this study, we chose the Plackett-Burman statistical designs, which allow for the screening of multiple factors and the determination of factors with significant positive or negative effects on production.

A matrix of 12 trials was studied and the protease activities are shown in (Table 15), and the statistical analysis of the production results was performed using Minitab software version 19.

Table15. Plackett–Burman design for the study of 11 factors with 12 experiments and protease production

Experiences	Variables											Protease (IU)
	A	B	(C)	D	E	(F)	G	H	(I)	J	(K)	
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	**49569,2**
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	**43876,9**
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	**49661,5**
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	**52216,8**
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	**42064,5**
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	**48276,9**
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	**45353,8**
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	**39538,5**
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	**42216,9**
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	**47607,7**
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	**50153,8**
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	**40769,2**

According to the statistical analysis (table15), the effect was calculated by changing the response as the factor changes from its lower (−1) level to its higher (+1) level using student's t-

test. The P–value of each factor was also evaluated. Factors with P-values less than 0.05 (P-value <0.05) were considered significant factors for protease production [8].

Protease production was influenced by factors with a significant positive or negative effect ($p \leq 0.05$) (table 3), namely B: Yeast extract (p=0.000), C: Corn steep liquor (p= 0.008), and J: tween 20 (p=0.017). The reduced polynomial equation of protease production (Y) is as follows:

$$Y = 45942 + 3639\,C4 + 1264C5 + 992C7 \quad (1)$$

Table.16 : Regression coefficients

Term	Coeff	Coef ErT	t-value	p-value	FIV
Constant	45942	253	181,47	0,000	
C1	140	253	0,55	0,609	1,00
C2	-104	253	-0,41	0,701	1,00
C4	3639	253	14,37	0,000	1,00
C5	1264	253	4,99	0,008	1,00
C7	992	253	3,92	0,017	1,00
C8	132	253	0,52	0,631	1,00
C10	-94	253	-0,37	0,729	1,00

The Fisher of the model F-value = 35.39 with p = 0.002 ($\leq$ 0.05) is very significant, allowing us to conclude that the model was adequate and that the production of the protease was well explained by humidity, inoculum, and incubation period. This is also supported by the value of R^2 (coefficient of determination) and R^2 adjusted of the model at 0.9841 and 0.9563 (a value > 0.75 indicates aptness of the model) This means that 98.41% of the protease production was influenced by the selected factors [8].

Table.17: Variance analysis of protease production from *Rhodotorula sp1*

Source	FD	Squares fit	Adjusted Mean Square	F-value	p-Value
Model	7	190538753	27219822	35,39	0,002
Linear	7	190538753	27219822	35,39	0,002
C1	1	235685	235685	0,31	0,609
C2	1	131016	131016	0,17	0,701
C4	1	158894097	15889409	206,6	0,000
C5	1	19163278	19163278	24,92	0,008
C7	1	11800734	11800734	15,34	0,017
C8	1	207978	207978	0,27	0,631
C10	1	105966	105966	0,14	0,729
Erreur	4	3076389	769097		
Total	11	193615143			

- **Pareto Charts**

The Pareto chart (Figure 14) graphically represents the results of the plan analysis. For protease production, the most significant effect is that of factor C (yeast extract), followed by factor D (corn steep liquor) and factor E (Tween 20).

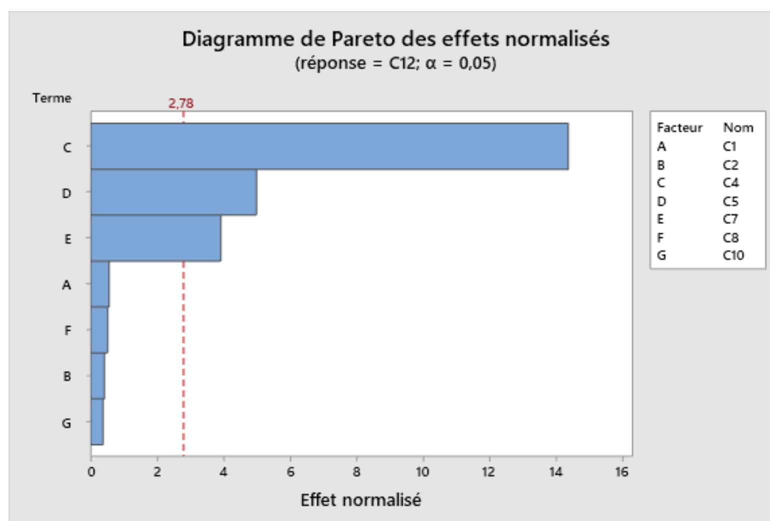


Figure.14: Pareto diagram of normalized effects

2.3.1. Model Validation

The production of *Rhodotorula sp1* protease reached 87538.46 IU, it doubled compared to the production before optimization (46584.61 IU). This confirms the effectiveness of Plackett and Burman's plans for improving microbial enzyme production.

2.3.2. Nitrogen Source

Yeast extract and corn steep liquor are sources of organic nitrogen that influence strain growth and enzyme production [74]. This implies that the medium does not contain enough nitrogen, which requires an additional source of organic nitrogen. NaNO3, is a source of inorganic nitrogen, its effect is not significant, this is probably explained by the fact that its addition resulted in an increase in the nitrogen level and probably in exceeding the critical threshold. Which causes an imbalance in the C/N ratio. In addition, yeast extract is also used by microorganisms as a source of vitamins.

2.3.3 Carbon Source

The effects of glucose and sucrose are negative on protease production because a concentration of 20% orange waste provides sufficient carbon for the growth of the strain and enzyme production.

2.3.4. Salts

MgSO4 has a negative effect on protease production because the chemical composition of the waste already contains enough of this salt, which is an activator for protease.

2.4 Characterization of the Protease

2.4.1. Effect of Temperature on Enzymatic Activity

Different temperatures ranging from 30°C to 80°C were studied on the activity of the protease from *Rhodotorula sp.*1 at pH 4.

The results in figure 15 show that the enzymatic activity increases with temperature up to the optimal temperature.

These results indicate that the protease produced by *Rhodotorula sp1* droopped at a temperature of 40°C and gave an activity level.

Luang *et al.* [75] showed that the optimal temperature for amylase of *Bacillussp.* 3.5AL2 was 60 °C; moreover, Finore et al. [76] obtained maximal α-amylase secretion from

Anoxybacillusamylolyticus at 60 °C. The results showed that *G. candidum* PO27 α-amylase is a thermophilic enzyme with potential use in industrial processes. Microorganisms able to grow optimally at temperatures between 50 °C and 60 °C are known as moderate thermophiles. It can be assumed that moderate thermophiles, which are closely related phylogenetically to mesophilic organisms, can adapt to life in warm environments

Josephine *et al*.,2012 They found that 40 °C is the ideal temperature for the protease produced from *Bacillus SNR01* Waheed *et al.*,2020. Note that the enzyme has demonstrated its maximum activity at 60 °C [75].

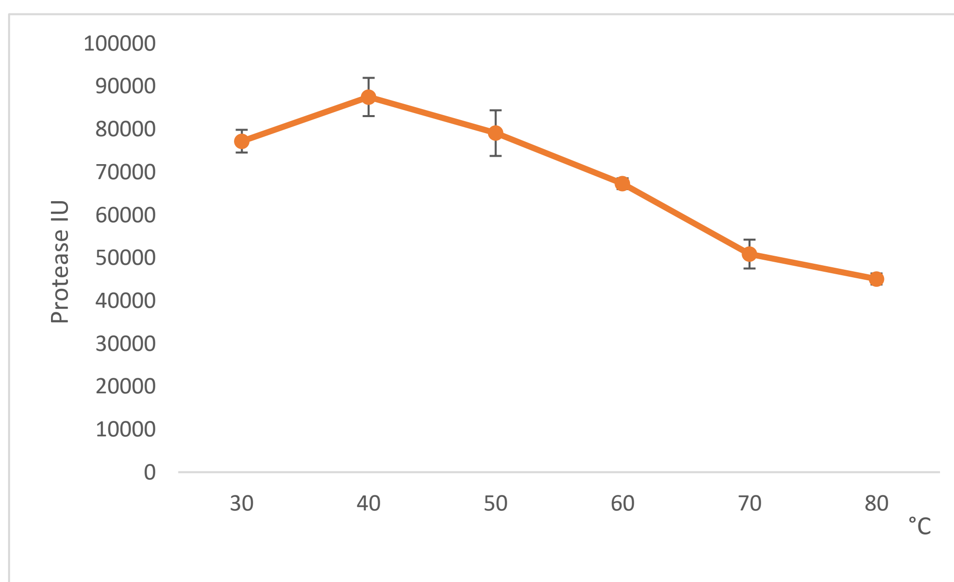


Figure.15: The enzymatic activity with different temperature.

2.4.2. Effect of pH on enzymatic activity

Different pHs were studied on the protease activity of *Rhodotorula sp1* : pH2, pH3,pH4 ,pH5,pH6,pH7.

The results in the figure 16 show that protease activity increases with pH up to the optimal pH (pH4) beyond which the activity decreases.

According to Gupta *et al.,* (2007), enzymes are sensitive to different pH levels and other functions over an electrolytic range.The extracellular acidic protease produced by *Rhodotorula glutinis*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida humicola, Rhodotorula mucilaginosa* L7 and *Rhodotorula oryzicola had* a pH optimum of 2.8 to 3.5, 4.3, 3.4 to 3.8, 4, 5 and 6.51, respectively [44, 75, 76].

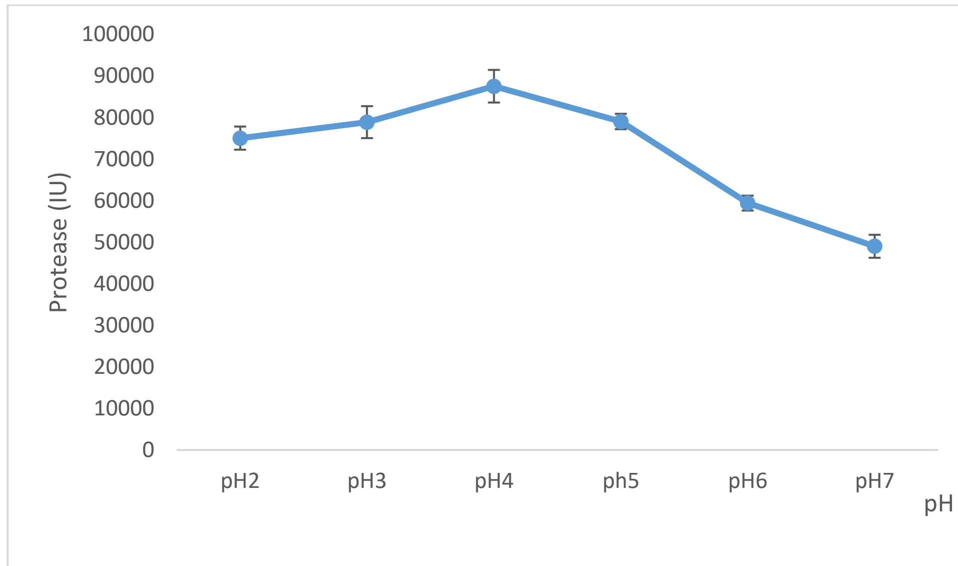


Figure 16: Protease activity with different pH

3. Effect of pepstatin on protease

The effect of pepstatin on the protease of *Rhodotorula sp1* was studied and the result is shown in the figure 17.

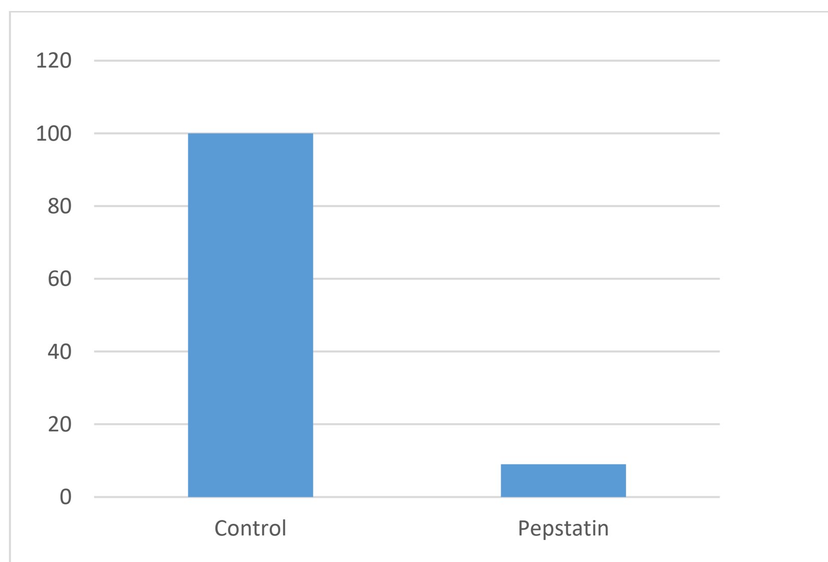


Figure.17: Effect of pepstatin on protease activity from *Rhodotorula sp1*

According to these results, the optimal pH for the activity of the protease from *Rhodotorula sp1* is pH 4 and pepstatin inhibited the protease activity. This allows us to conclude that *Rhodotorula sp1* protease is an acidic protease.

Conclusion

Conclusion

This study showed that yeast isolated from honey from Collo, Wilaya of Skikda. was identified by the study of morphological characters (macroscopic and microscopic) and biochemical characters by the APi 32C galleries. Its code of 5461 7401 17 belongs to the genus Rhodotorula.

This proteolytic yeast has shown its ability to grow on various agro-food waste, namely: whey, date waste, orange peel, wheat bran and tomato waste. *Rhodotorula sp1* produces acidic, neutral and alkaline protease on different media. The best fermentation substrate is 20% orange peel. However, the activity at pH 4 is better in the presence of orange waste (peel) (11815 IU), followed by date waste (6738 IU) and whey (4800 IU). And for significant activity (11815 ± 1205.66 IU) of acid protease (pH4).

Optimization of protease production was achieved by applying the Plackett and Burman plans. 07 factors were studied such as glucose, sucrose, yeast extract, corn steep liquor, $MgSO_4$, tween 20 and $NaNO_3$ using the matrix of 12 tests. Statistical analysis revealed that yeast extract ($p=0.000$), corn steep liquor ($p=0.008$) and tween 20 ($p=0.017$) are the factors with a significant positive effect on protease activity. The $R^2 = 0.9841$ and The Fisher of the model F-value = 35.39 with $p = 0.002$ (≤ 0.05) is very significant, allowing us to conclude that the model was adequate and that the production of the protease was well explained by studied factors. Production in liquid fermentation is carried out using 20% orange peel and 20% yeast, corn steep liquor and 20% tween for 48 hours at 30°C and 150rpm. The results indicated that protease synthesis doubled. This confirms that Plackett and Burman's plans constitute an effective tool for optimizing microbial enzyme production.

The study of the characteristics of the enzyme showed that *Rhodotorula sp1* protease has an optimal temperature of 40°C, an optimum pH of 4 and it is inhibited by pepstatin. This allows us to conclude that *Rhodotorula sp1* protease is an acidic protease.which was important for industrial use.

In perspective, it would be interesting to:

-Optimize the environment using the CCD coupled with RSM.

-Purified the enzyme and study other characteristics.

-Try several applications of the enzyme.

-Study the kinetics of the enzyme.

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ANNEX

ANNEX 1 : <u>Buffer preparation</u>

Buffer preparation

Ph= 04

_Citric acid ………………………………………………………………..0.1M($C_6H_8O_7$).

_Disodium phosphate……………………………………………0.2M (Na_2HPO_4).

_Distilled water.

Ph=07

_Monosodium phosphate ………………………………………0.2M (NaH_2PO_4).

_Disodium phosphate………………………………………………2M (Na_2HPO_4).

_Distilled water .

Ph=09

_Glysin ……………………………………………………………………0.2M ($C_2H_5NO_2$).

_NaOH………………………………………………………………………0.2M.

_Distilled water.

_Titration using a ph mater (previously calibrated) to the ph of interest .

ANNEX 2 : Tyrosine standard curve protocol

Table 01 : Tyrosine standard curve protocol

Tubs N°	Control	01	02	03	04	05
Concentration (µg/ml)	0	20	40	60	80	100
Tyrosine solution (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
Na_2 CO_3 (2%in NaOH 0.1N)	2.5	2.5	2.5	2.5	2.5	2.5

_ Shaking and incubation at room temperatue for 10 minutes.

_Add 2.5 (ml) diluted folin 1\2 to all tubs .

_Incubation in the dark for 30 minutes .

_Optimal density reading at 750nm**.**

Abstracts

Résumé

Cette étude vise la production de protéases par la levure Rhodotorula sp1 isolée du miel et identifiée par des caractéristiques morphologiques et biochimiques

Différents déchets agroalimentaires à savoir les déchets de tomate, les dattes déclassées, le lactosérum, la peau d'orange et le son de blé pour la culture de la levure par fermentation liquide pendant 28 heures à une température de 30°C. Les résultats ont révélé que la peau d'orange représente le meilleurs substrat pour *Rhodotorula sp1*. La souche a montré une meilleur production de la protéase acide (à pH4). L'effet de la concentration du déchet d'orange sur la production protéasique a été étudié et il s'est avéré que la concentration de 20% est la meilleure. Afin d'améliorer la production enzymatique le modèle expérimental de Plackett-Burman a été appliqué. Les facteurs testés comprenaient le glucose, le saccharose, l'extrait de levure, le corn steep liquor, le Tween 20, le $NaNO_3$ et le MgSO4.

Les facteurs ayant un effet positif ou négatif significatif étaient le Tween 20 ($p = 0,017$), l'extrait de levure ($p = 0,000$) et le corn steep liquor ($p = 0,008$). Ces facteurs ont augmenté la production d'activité enzymatique de 46 584,61 UI à 87 538,46 UI. La caractérisation de la protéase de *Rhodotorula sp1* a indiquée que sa température optimale est de 40 °C et le pH optimal pour la production était de 4. Il s'est avéré aussi que la protéase a été inhibée par la pepstatine, confirmant sa nature acide. La protéase de *Rhodotorula sp1* peut donc être utilisée dans divers secteurs industriels, notamment dans l'industrie agroalimentaire.

Mot clés : Protéase acide , *Rhodotorula sp* , levure, Optimisation, déchet agro-alimentaire.

Abstract:

This study aims at the production of proteases by the yeast *Rhodotorula sp1* isolated from honey and identified by morphological and biochemical characteristics.

Different agri-food waste, namely tomato waste, downgraded dates, whey, orange peel and wheat bran for the cultivation of yeast by liquid fermentation for 28 hours at a temperature of 30°C. The results revealed that orange peel represents the best substrate for *Rhodotorula sp1.* The strain showed better production of acid protease (at pH4). The effect of the concentration of orange waste on protease production was studied and it turned out that the concentration of 20% is the best. In order to improve enzyme production the Plackett-Burman experimental model was applied. Factors tested included glucose, sucrose, yeast extract, corn steep liquor, Tween 20, $NaNO_3$ and $MgSO_4$.

Factors with a significant positive or negative effect were Tween 20 ($p = 0.017$), yeast extract ($p = 0.000$) and corn steep liquor ($p = 0.008$). These factors increased the production of enzyme activity from 46,584.61 IU to 87,538.46 IU. Characterization of the Rhodotorula sp1 protease indicated that its optimum temperature is 40°C and the optimum pH for production was 4. It was also found that the protease was inhibited by pepstatin, confirming its acidic nature. . *Rhodotorula sp1* protease can therefore be used in various industrial sectors, particularly in the food industry.

Keywords: Acid protease, *Rhodotorula sp*, yeast, Optimization, agro-food waste.

ملخص

تهدف هذه الدراسة إلى إنتاج البروتياز بواسطة خميرة *Rhodotorula sp 1* المعزولة من العسل والمحددة بالصفات المورفولوجية والكيميائية الحيوية.

المخلفات الزراعية الغذائية المختلفة وهي مخلفات الطماطم والتمر المخفض ومصل اللبن وقشر البرتقال ونخالة القمح المستخدمة في زراعة الخميرة عن طريق التخمير السائل لمدة 28 ساعة عند درجة حرارة 30 درجة مئوية. أظهرت النتائج أن قشر البرتقال يمثل أفضل ركيزة لنبات *Rhodotorula sp 1*. أظهرت السلالة إنتاجًا أفضل للبروتياز الحمضي (عند الرقم الهيدروجيني 4). تمت دراسة تأثير تركيز مخلفات البرتقال على إنتاج الأنزيم البروتيني وتبين أن تركيز 20% هو الأفضل. من أجل تحسين إنتاج الإنزيم تم تطبيق نموذج بلاكيت-بورمان التجريبي. وشملت العوامل التي تم اختبارها الجلوكوز والسكروز ومستخلص الخميرة ومحلول الذرة الحاد وتوين 20 و$NaNO_3$ و$MgSO_4$.

وكانت العوامل ذات التأثير الإيجابي أو السلبي الكبير هي توين 20 ($p=0.017$)، ومستخلص الخميرة ($p= 0.000$) ومحلول الذرة شديد الانحدار ($p=0.008$). أدت هذه العوامل إلى زيادة إنتاج نشاط الإنزيم من 46,584.61 وحدة دولية إلى 87,538.46 وحدة دولية. أشار توصيف بروتياز *Rhodotorula sp 1* إلى أن درجة الحرارة المثلى له هي 40 درجة مئوية وأن الرقم الهيدروجيني الأمثل للإنتاج هو 4. كما وجد أن البروتياز تم تثبيطه بواسطة البيبستاتين، مما يؤكد طبيعته الحمضية. ولذلك يمكن استخدام البروتياز *Rhodotorula sp 1* في مختلف القطاعات الصناعية، وخاصة في صناعة المواد الغذائية.

الكلمات المفتاحية: حمض البروتياز، *Rhodotorula sp*، الخميرة، التحسين، مخلفات الأغذية الزراعية.

Presented by: Mermoul Kaouther
Lahlou Naziha

Study of the optimization of protease production in Rhodotorula SP1 by placket and burman plans

Thesis submitted for the obtention of Master degree in Biochemistry

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Keywords: Acid protease, *Rhodotorula sp*, yeast, Optimization, agro-food waste.

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