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Acid proteases production by *Rhodotorula sp*2: study of the optimization of the production.

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To my greatest supporters and sources of inspiration, I dedicate this work with all my infinite love and gratitude

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To the one who made me a strong woman, my source of life, love and affection, to my support who was always by my side to support and encourage me, to my prince dad (KHOUDJA)

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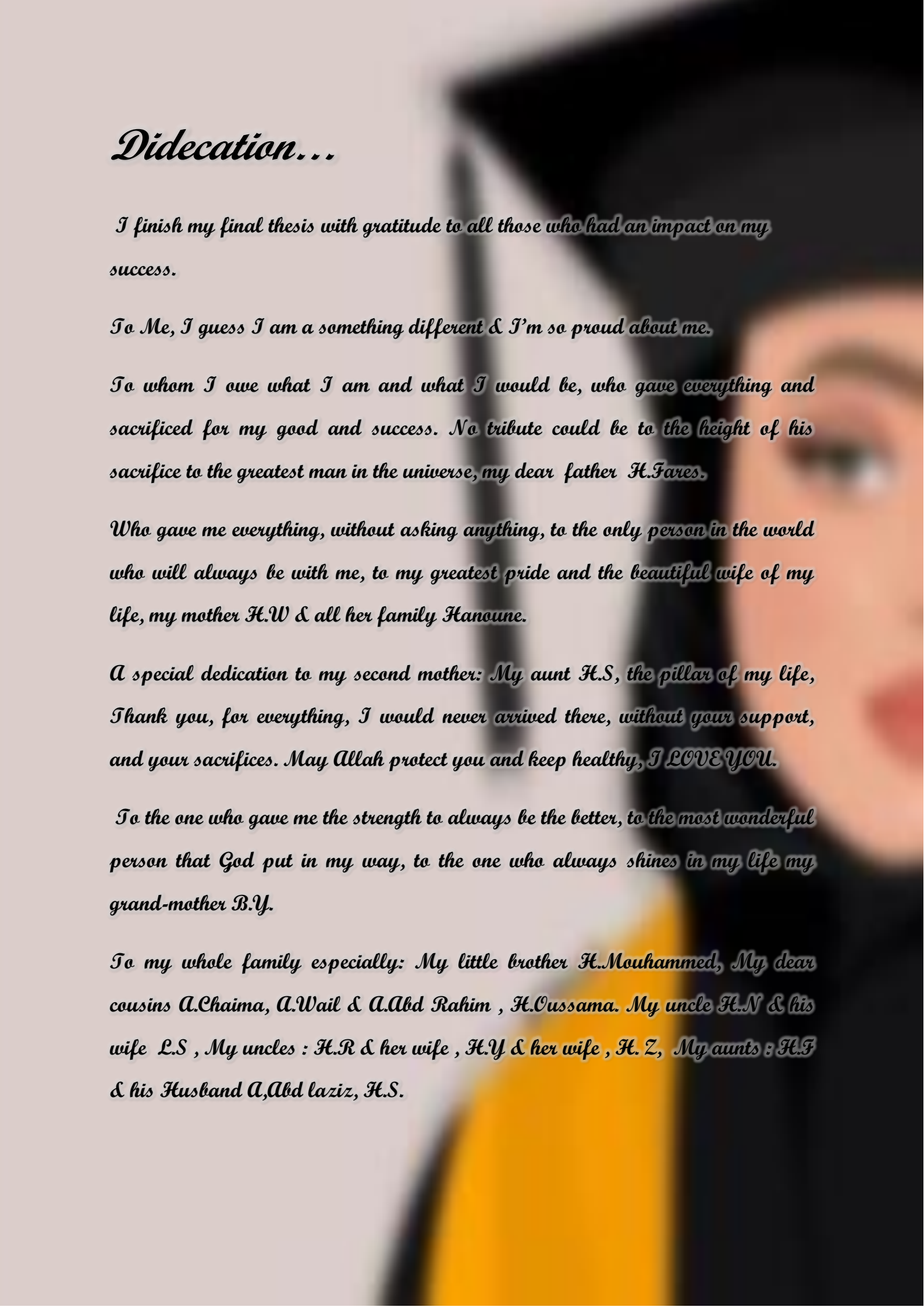
♡ AMMARI Asma ♡



Didecation…

I finish my final thesis with gratitude to all those who had an impact on my success.

To Me, I guess I am a something different & I'm so proud about me.

To whom I owe what I am and what I would be, who gave everything and sacrificed for my good and success. No tribute could be to the height of his sacrifice to the greatest man in the universe, my dear father H.Fares.

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Lists

Shortlist

- **Aw:** Water activity.
- **AE:** Enzyme activity.
- **CCD**: Central Composite Design
- **FML** Fermentation in liquid medium.
- **FMS:** Fermentation in Solid Medium.
- **PBD:** Plackett-Burman Design.
- **FOAT:** One factor at a time.
- **TCA:** Trichloroacetic acid.
- **UI:** International Unity.
- **YPGA:** Yeast Peptone Glucose Agar.

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Abstract

Abstract

The aim of the work is a study of the production of a protease by culture of a yeast strain *Rhodotorula sp2* on liquid medium (FML) at different pH (acid, neutral and alkaline). Five substrates are tested: tomato waste, orange waste, date waste, wheat bran and whey with a seeding rate of 10^7 cells at 30°C. Initially we start by determination of concentration of the different substrates tested that gives maximum protease production. The statistical method of one factor at a time (OFAT) showed that the best proteolytic activities are obtained in liquid fermentation based on orange waste at pH 4 (acid protease). In a second step, the statistical plans of Plackett and Burman are respectively used to determine and optimize the factors influencing the production of acid protease. Culture media are prepared using a factorial design of Plackett-Burman N=12 trials and N-1 factors: seven real factors (Glucose, Sucrose, Yeast extract, Corn-steep Liquor, Tween80, Na_2 CO_3, Mg SO_4) and four errors. The results obtained show that the significant factors influencing the enzyme production by the yeast *Rhodotorula sp2* are: sucrose and yeast extract. The optimal values of the variables studied are determined by the response surface methodology namely: sucrose 1% and yeast extract 2%. The validation of the model allowed to obtain an acid protease activity of 17555.55 IU/ml, which is 2 times higher than under non-optimized conditions. These significant results confirm the importance of statistical design in acid protease production.

Keywords: Acid Protease, *Rhodotorula sp*, FML, Tomato waste, Orange waste, Date waste, Wheat bran, Whey, Optimization.

Résumé

L'objectif de ce travail est d'étudier la production d'une protéase par culture d'une souche de levure Rhodotorula sp2 sur milieu liquide (FML) à différents pH (acide, neutre et alcalin). Cinq substrats sont testés : les déchets de tomates, les déchets d'oranges, les déchets de dattes, le son de blé et le lactosérum avec un taux d'ensemencement de 107 cellules à 30 °C. Dans un premier temps, nous commençons par déterminer la concentration des différents substrats testés qui donne une production maximale de protéase. La méthode statistique d'un facteur à la fois (OFAT) a montré que les meilleures activités protéolytiques sont obtenues en fermentation liquide à base de déchets d'orange à pH 4 (protéase acide). Dans un second temps, les plans statistiques de Plackett et de Burman sont respectivement utilisés pour déterminer et optimiser les facteurs influençant la production de protéase acide. Les milieux de culture sont préparés en utilisant un plan factoriel des essais Plackett-Burman N=12 et des facteurs N-1 : sept facteurs réels (glucose, saccharose, extrait de levure, liqueur de maïs, Tween80, Na2 CO3, Mg SO4) et quatre erreurs. Les résultats obtenus montrent que les facteurs significatifs influençant la production d'enzymes par la levure Rhodotorula sp2 sont : saccharose et extrait de levure. Les valeurs optimales des variables étudiées sont déterminées par la méthodologie de surface de réponse à savoir : saccharose 1% et extrait de levure 2%. La validation du modèle a permis d'obtenir une activité protéase acide de 17555,55 UI/ml, soit 2 fois plus que dans des conditions non optimisées. Ces résultats significatifs confirment l'importance du plan statistique dans la production de protéases acides.

Mots-clés : Protéase acides, *Rhodotorula sp*, FML, Déchets des tomates, Déchets d'oranges, Déchets des dattes, Son de blé, Lactosérum, Optimisation.

ملخص

الهدف من هذا العمل هو دراسة إنتاج البروتياز عن طريق زراعة سلالة الخميرة Rhodotorula sp2 على الوسط السائل (FML) عند درجة حموضة مختلفة (الحمض والمحايد والقلوي). يتم اختبار خمس ركائز: نفايات الطماطم، نفايات البرتقال، نفايات التمر، نخالة القمح ومصل اللبن بمعدل بذر 107 خلايا عند 30 درجة مئوية. أولاً، نبدأ بتحديد تركيز الركائز المختلفة التي تم اختبارها والتي تعطي أقصى إنتاج من البروتياز. أظهرت الطريقة الإحصائية لعامل واحد في كل مرة (OFAT) أن أفضل أنشطة انحلال البروتين يتم الحصول عليها في التخمير السائل بدءًا على نفايات البرتقال عند الرقم الهيدروجيني 4 (البروتياز الحمضي). في الخطوة الثانية، يتم استخدام الخطط الإحصائية لبلاكيت وبورمان على التوالي لتحديد وتحسين العوامل التي تؤثر على إنتاج البروتياز الحمضي. يتم إعداد الوسائط الثقافية باستخدام تصميم مضروب لـ Plackett-Burman N = 12 مقايسة وعامل N-1: سبعة عوامل فعلية (الجلوكوز والسكروز ومستخلص الخميرة وخمور الذرة Tween80 Na2 CO3 و Mg SO4) وأربعة أخطاء. تظهر النتائج التي تم الحصول عليها أن العوامل المهمة التي تؤثر على إنتاج الإنزيمات بواسطة الخميرة Rhodotorula sp2 هي: مستخلص السكروز والخميرة. يتم تحديد القيم المثلى للمتغيرات التي تمت دراستها من خلال منهجية سطح الاستجابة وهي: السكروز 1٪ ومستخلص الخميرة 2٪. سمح التحقق من صحة النموذج بالحصول على نشاط بروتياز حمضي 17555,55 IU/ml، وهو أكثر 2 مرة من الظروف غير المثلى. تؤكد هذه النتائج المهمة أهمية الخطة الإحصائية في إنتاج البروتياز الحمضي.

الكلمات الرئيسية: FML، Rhodotorula sp، Acid Protease، نفايات الطماطم، نفايات البرتقال، نفايات التاريخ، نخالة القمح، مصل اللبن، التحسين.

الكلمات الرئيسية: حمض البروتياز، FML، Rhodotorula sp، نفايات الطماطم، نفايات البرتقال، نفايات التمر، نخالة القمح، مصل اللبن، التحسين.

Introduction

Introduction

The worldwide industrial enzyme market is estemed at $6,429.50 million in 2021 and is anticipated to reach an esteem of $9,276.34 million by 2028, at a compound yearly development rate (CAGR) of 6.30% over the estimate period, 2022-2028 *(Gaurkhede, 2024).*

The utilize of enzymes makes a difference to guarantee way better item quality as well as decreased squander, generation costs and vitality utilization, which fortifies the request for mechanical proteins amid the arranged plan. The developing request for mechanical chemicals extricated from the nutraceutical and nourish businesses is anticipated to invigorate the development of the showcase for mechanical chemicals within the close future. In expansion, buyers are progressively wellbeing cognizant, which fortifies the utilization of useful nourishment items, hence expanding the demand for enzymatic items within the coming a long time *(Gaurkhede, 2024).*

Proteases are considered as one of the foremost vital bunches of mechanical chemicals, speaking to approximately 60 % of the worldwide advertise share. Other than cleanser, food businesses are the most application of proteases, where they are utilized in preparing, meat tenderization, synthesis of aspartame, arrangement of protein hydrolysates, and dairy fabricating *(Aljammas et al., 2022).*

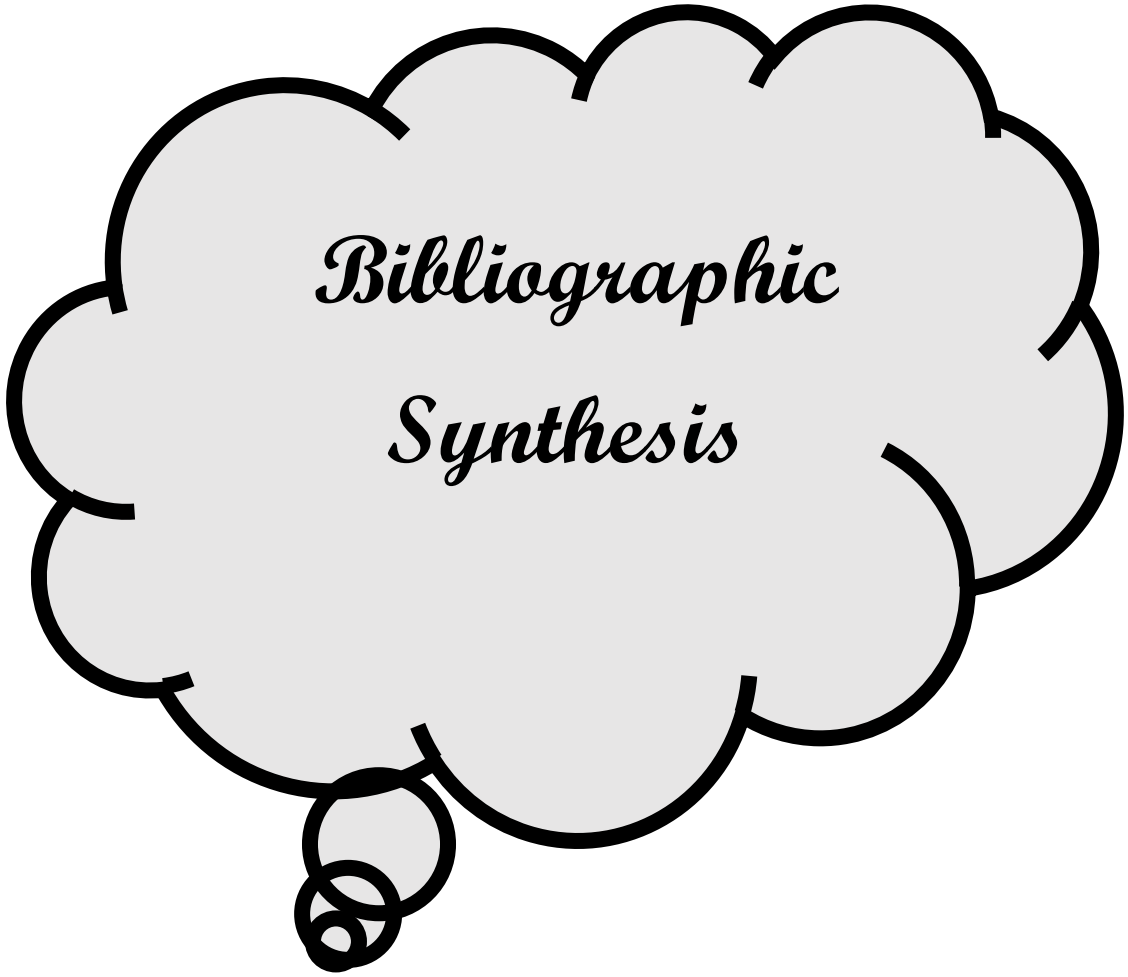
Microbial proteases are among the foremost imperative and broadly considered hydrolytic enzymes since the starting of enzymology. They constitute more than 40% of the total worldwide generation of enzymes. They are delivered by a huge number of organisms, counting bacteria, fungi and yeasts *(Adetunji et al., 2023). Rhodotorula sp* is one of the main fungal species applied in the commercial production of enzymes by submerged fermentation process *(Oliveira et al., 2021).*

Proteolytic enzymes can be produced by liquid as well as solid fermentation *(De castro et al., 2014).* The cost of producing commercially available enzymes by the submerged fermentation is generally too high for biotechnology applications to this reason solid fermentation is an alternative technique for the production of enzymes industrial *(Mais, 2018).* Fungal cultures are carried out either on solid substrate (FMS) or in submerged culture (FML) to produce metabolites (enzymes). Solid cultures are preferred for the following reasons: simplicity of the process, low cost and enzyme yields are more important *(De casro et al., 2014).*

The submerged fermentation is generally performed at the sight of a free liquid in which soluble substrates are and is the most commonly used technology for the huge range of enzyme production *(Namnuch and Thammasittirong, 2020).*

On the other hand, most proteases are produced either by mold or by bacteria. The aim of the study is to exploit the enzymatic potential of yeasts, because industry it is interesting to use new molecules that meet industry criteria. The different stages of this work are:

- Reactivation of the yeast selected for protease production.
- Conduct of fermentation.
- Optimization of acid protease production by applying the two statistical plans: O.F.A.T and Plackett-Burman.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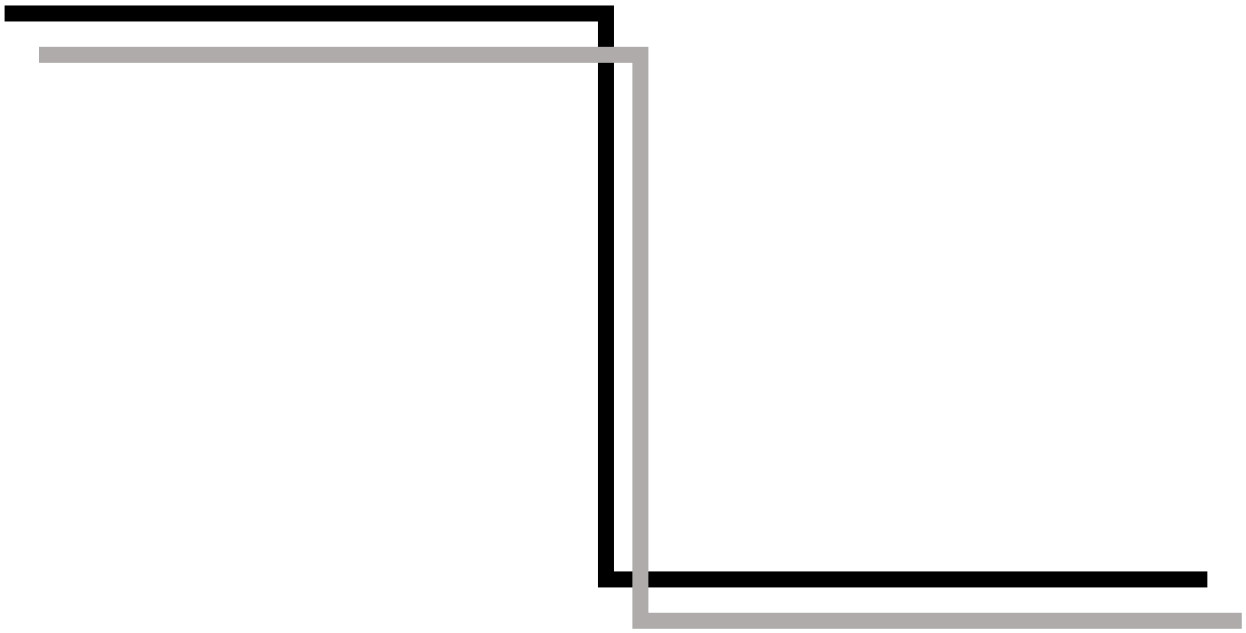
Chapter 01



...Yeasts...

1. Generalities

Yeasts are eukaryotic microorganisms, single-celled thallus parasites still, photosynthetic and chemo-heterotrophic *(Kurtzman et al., 2011)*. They are utilized in a few mechanical, biotechnology, therapeutic and natural divisions (**Figure 01**) *(Ahasanul et al., 2020)*.

A few yeasts have a sexual propagation that compares to a stage of their cycle natural stage substituting haploid and diploid. Duplication agamic is continuously display, is basically done by budding at the closes of the huge tomahawks of cells on the off chance that they are ovoid or stretched *(Bennamoun, 2017)*.

Yeast ageing plays a central part within the discharge of a number of compounds such as: Esters, Lactones, Thiols, Alcohols and phenolic compounds included within the improvement of beer's characteristic enhance *(Carrau et al., 2015)*.

The advantages of utilizing yeasts over other micro-organisms are:

- The tall emission capacity.
- Tall development rate on a wide assortment of carbon source.
- The capacity to create numerous post-translation changes.
- Simple to develop in little vessels or huge bioreactors.
- Simple refinement of the item and need of affectability to irresistible operators such as bacteriophages *(Wagner and Alper, 2016)*.

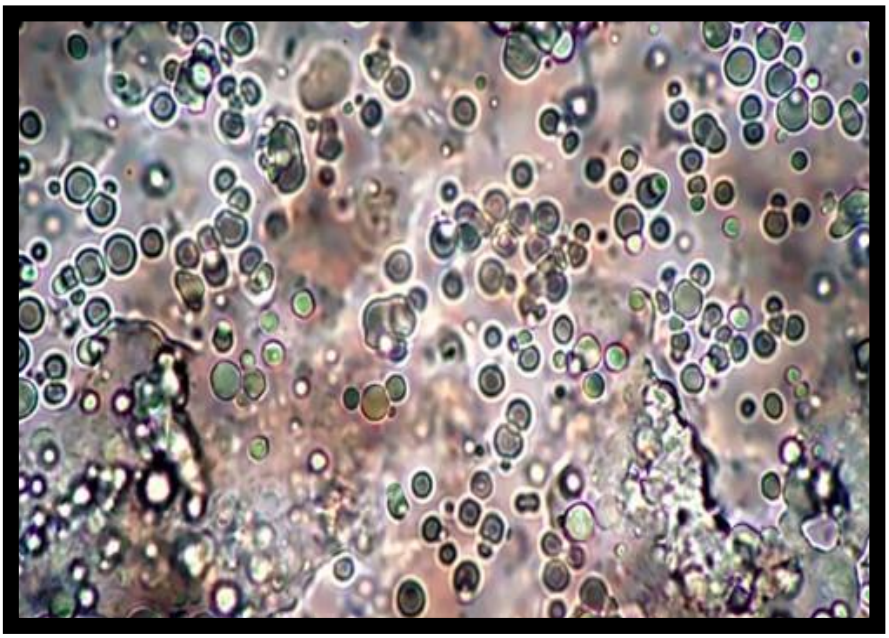


Figure 01: Yeast cell under the microscope **(www.iStoke.com).**

2. Characteristics of yeasts

2.1. Morphological characteristics

Yeasts speaking to the best eukaryotes seen their cell composition. They are made up of cell shells, a cytoplasm containing different organelles, and a chromosome core (**Figure 02**) *(AL Daccache, 2019).*

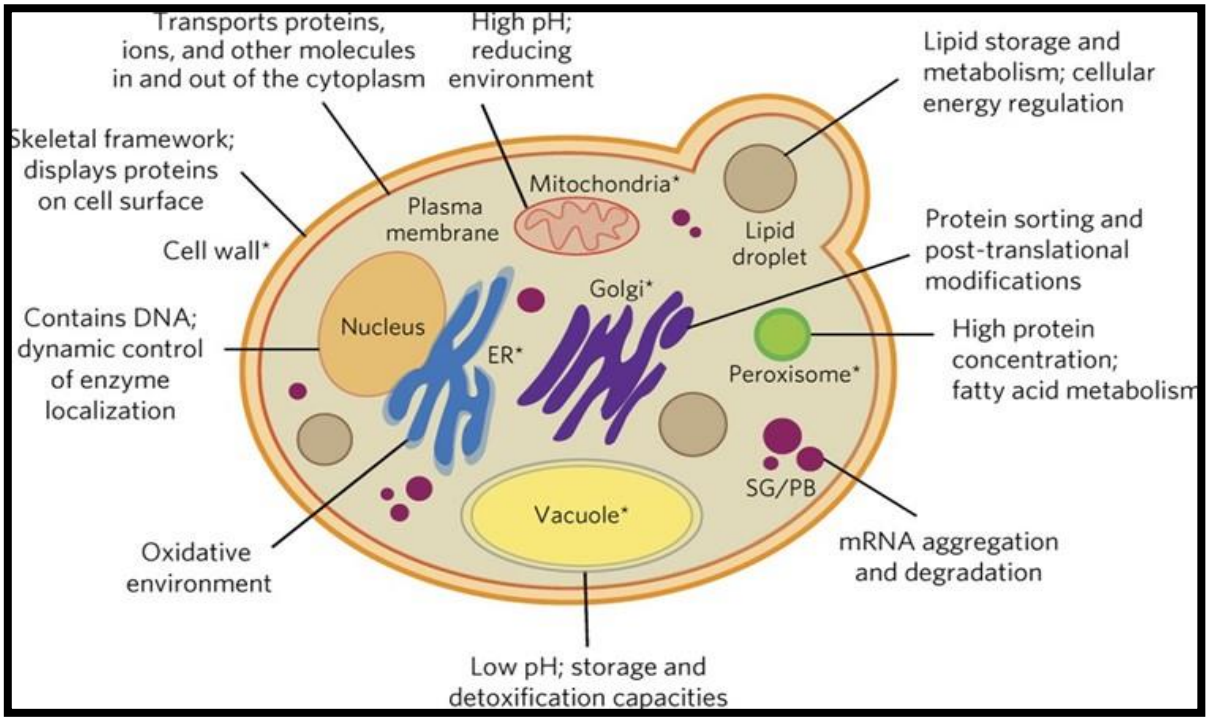


Figure 02: Structure of yeasts *(Hammer and Avalos, 2017).*

Yeast cells are ordinarily ovoid with an outside cell divider and an inner layer encompassing its intracellular environment *(Giulia et al., 2021)*. In any case fundamentally, their cell measure ranges from a number of microns to 25 to 30 microns in ovoid or circular shape *(Haroldo et al., 2020)*. the interior of the yeast is compartmentalized of a core, a few vacuoles, mitochondria, endoplasmic reticulum, and a number of vesicular bodies *(Thumm et al., 2000; Li et al., 2009).*

2.2. Sexual characteristics

Sexual characteristics include nearness of the ascus and ascospores. The mode of ascus arrangement is characteristic of haploid and homothallic species. Ascospores are display in ascus. *Kluyveromyces, Hansenula, Pichia* appear nearness of ascospores.

The basidiomycetous yeast genera appear arrangement of teliospores for illustration, *Rhodosporidium*. The spores are thick walled, habitually wealthy in lipid, perhaps fuscous or spheroidal (**Figure 03**) *(Phale, 2018).*

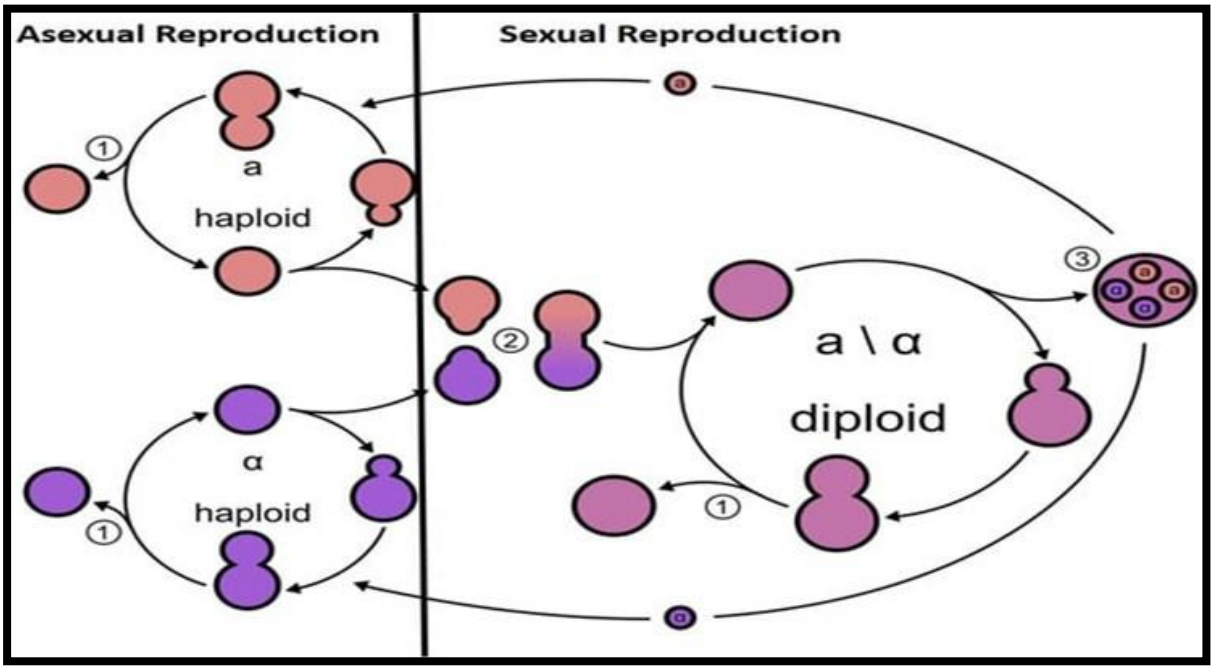


Figure 03: Schematic of the life cycle of yeasts *(Jane, 2019).*

2.3. Cultural Characteristics

In case of social characteristics, development in fluid media and strong media is watched. Yeast developed in fluid media may result within the arrangement of silt, a ring, a pellicle. Pellicle arrangement on a liquid medium may be a property related with oxygen request of yeast. Development on strong media possibly either mucoid, butyrous, friable, pigmented and tangle. Lipomyces appears characteristically mucoid development. Shade is for occasion profoundly characteristic of genera *Rhodotorula* and *Sporobolomyces (Phale, 2018).*

2.4. Physiological characteristics

The capacity or failure to ferment carbohydrates into ethanol and carbon dioxide by yeasts is the foremost critical to distinguish species. An assortment of sugars is matured by an assortment of yeast. *Saccharomyces* is known to appear maturation overwhelming whereas genera like *Rhodotorula* and *Lipomyces* are entirely no fermentaries *(Sonali et al., 2018).*

The capacity or failure to utilize diverse sources of nitrogen can be utilized to classify yeasts. With the exemption of the class Saccharomyces which develops as it were in media containing certain yeast or protein hydrolysates, all yeasts may utilize an assortment of nitrogen sources *(Sonali et al., 2018).*

Most yeasts deliver follows of unstable and non-volatile acids. We see this when overabundance acidic corrosive is delivered. A few species of *Kloeckera, Hansenula, Trichosporon* are known to deliver impressive sums of acids. In fitting culture conditions, a few yeast strains create polysaccharides extracellular. *Bullera, Trichosporon* produces certain amyloid compounds *(Sonali et al., 2018).*

3. Reproduction

3.1. Asexual reproduction

Asexual reproduction is accomplished by budding. A little striking mass shows up on the surface of the mother cell that develops small by small and after that withdraws when it comes to the same measure. At the same time, the core of the mother cell moves towards the Fringe, it extends, a portion penetrates into the bud. It at that point isolates from the mother cell and some of the time clearing out a little "scar". The girl cell in this way delivered develops to reach approximately a breadth rise to two-thirds of the parent cell, at that point gives in turnmodern buds (**Figure 04**) *(Guinet et al., 1993).*

Beneath ideal developing conditions, this populace pairs each ninety minutes, which speaks to a record time for eukaryotic cells *(Silar et al., 2013).*

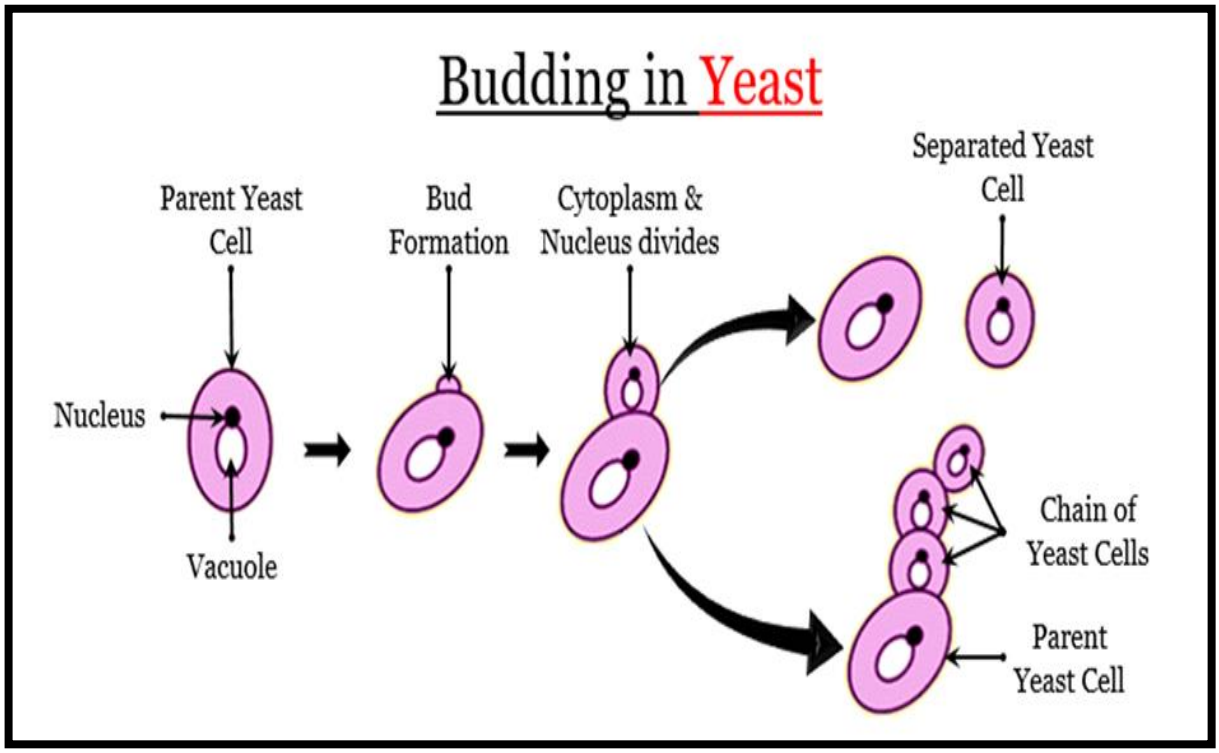


Figure 04: Asexual reproduction of yeast *(Ocean property, 2021).*

3.2. Sexual reproduction

In an ominous environment (tall in acetic acid derivation, moo in supplements, temperatures extremes…), the yeast diploid cell will sporulate deliver 4 or 8 cells haploid, called "ascospores" in ascomycetes, and "basidiospores" in basidiomycetes, which is able stay in life moderated down (**Figure 05**) In the event that natural conditions return favorable, spores are discharged, will grow, develop and start a unused vegetative proliferation cycle in haploid or diploid shape *(Montelh et al., 2012).*

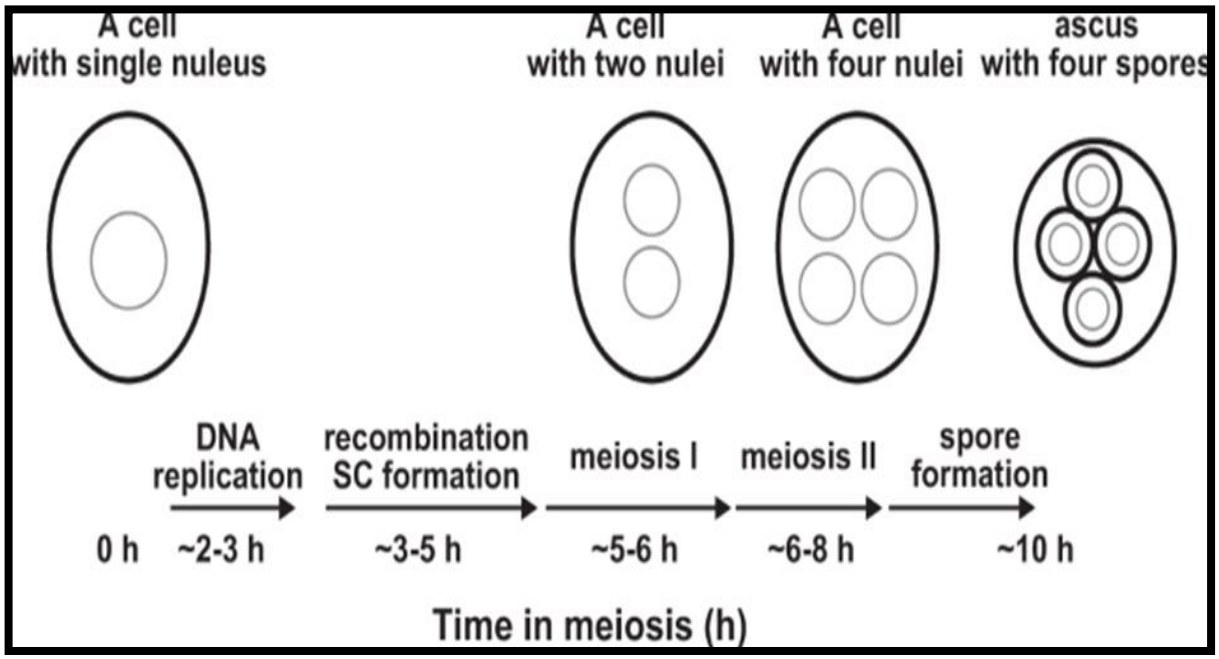


Figure 05: Sexual reproduction of yeast *(Takagi et al., 2021).*

4. Classification

Scientific classification is the characterization and refinement of taxa (species) based on the perception of particular characters. It is to recognize people who see just like the point to make a homogeneous set by common characters and varying from other sets *(Kiffer and Morelet 1997).* In specific unused ordered criteria such as composition based on DNA, divider structure, sort of coenzyme is taken into consideration to permit bigger ponders.

Depending on the mode of generation, yeasts can be classified into three classes:

4.1. Ascomycota (Ascomycetes)

Ascomycetes replicate through a sexual handle shape the gather of eumycete that contains the most prominent number of species, their meioscpores are created at interior a sack or asque (thus the title ascospores).

4.2. Basidiomycota

Basidiomycetes have sexual generation, around a third predominant species of organisms. They are characterized by the reality that meiofpores are outside called basidiospores.

4.3. Deuteromycetes

A set of yeasts gathering the bio genetic shapes of Dicaryomycota, does not duplicating by vegetative propagation. These are Ascomycetes at the anamorphic organize (Agamic); a few are basidiomycetes *(Tortora et al., 2007).*

5. *Rhodotorula* yeast

Rhodotorula yeast, an unmistakable microorganism competent of creating β-carotene, toluene, and torularhodin, can be found in different situations such as water, plants, creatures, and human living spaces (**Figure 06**) *(Hu et al., 2022).*

Compared to plant-based or chemical amalgamation strategies, *Rhodotorula* yeast offers a few preferences as a carotenoid maker. Its tall development rate and shorter generation cycle decrease development zone, water utilization, fetched, and natural affect. Also, *Rhodotorula* yeast can utilize low-cost agrarian and agro-industrial squander like corn cobs, straws, and molasses as a source of basic carbon and nitrogen, anticipating squander amassing and profiting the environment. These benefits make *Rhodotorula* yeast reasonable for applications within the nourishment, pharmaceutical, corrective, chemical, mariculture, and natural security businesses *(Thumkasem et al., 2023).*

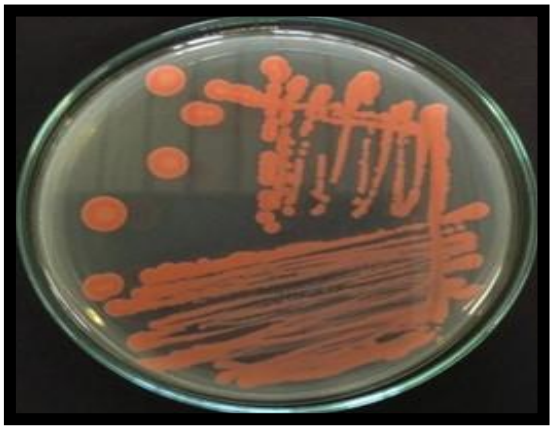


Figure 06: *Rhodotorula sp (Hu et al., 2022).*

5.1. Genus

Rhodotorula may be a common natural yeast that's found in discuss, soil, lakes, sea water, drain, and natural product juice. *Rhodotorula* species, portion of the Basidiomycota phylum, colonise plants, people, and other warm blooded creatures. The class *Rhodotorula* incorporates eight species, of which R. *mucilaginosa*, R. *glutinis*, and R. *minuta* are known to cause infection in people. *Rhodotorula* produces pink to ruddy colonies and blastoconidia that are unicellular missing pseudohyphae and hyphae. A few creators have separated *Rhodotorula* in distinctive biological systems and situations as well as depicted contaminations in creatures *(Wirth and Goldani, 2012).*

5.2. Taxonomy

The classification of yeasts is traditionally based on their physiological profiles and biochemical, molecular methods have been successfully applied to typing and identification of

yeast strains *(khater et al., 2019)*. The phylogenetic classification of *Rhodotorula* sp yeast *(http://ncbi.nlm.nih.gov/Taxonomy/Broswer., 2020)* is presented as follows (**Table 01**):

1. **Super kingdom**	• Eukaryota
2. **kingdom**	• Fungi
3. **Phylum**	• Basidiomycota
4. **Class**	• Microbotryomycetes
5. **Order**	• Sporidiobolales
6. **Family**	• Sporidiobolaceae
7. **Genus**	• *Rhodotorula*
8. **Spcies**	• *Sp*

5.3. Habitat

Rhodotorula species are omnipresent and basidiomycetous yeasts that can be found in discuss, soil, lakes, seawater, drain and natural product juice. These yeasts too have been found in human skin, nails and conjunctives, respiratory, gastrointestinal and urinary mucosa. In later a long time, it has risen as an artful pathogen, especially in immunocompromised patients due to maladies or drugs, central venous catheters (CVC) and other intrusive gadgets. There are few cases of fungemia detailed in immunocompetent patients *(Pereira et al., 2016).*

5.4. Morphology

It may be a heterothallic fungus whose hyphae are with nodular septa but it depends on the sex of these yeasts. The terminal telospores are solitary and nearly round with width 6.0 to 12.8 µm and length from 7.6 to 13.6 µm and granules. Metabasids are unicellular or bicellular. Basidiospores are ovoid, sessile and terminally shaped in metabasids *(Pereira et al., 2016).*

5.5. Nutritional requirements

Yeasts, like all mushrooms, are heterotrophic and thus require natural carbon. A few are fulfilled with glucose, others moreover require vitamins, amino acids... Their nitrogen needs are for the most part secured by salts ammonium, but other substances can be utilized: amino acids, urea..., or indeed nitrates (Hansenula case). Yeasts can, depending on the species, advantage from different carbohydrates, but they are seldom able to assault polyosides (Inulin, Starch).

- ✓ Source of carbon and energy
- ✓ Nitrogen source.
- ✓ Trace elements and vitamins.
- ✓ Temperature.
- ✓ pH.
- ✓ Respiration and fermentation.
- ✓ Osmotic pressure and water activity (Aw).

a. Carbon source and energy

Yeasts require carbon sources and antecedents for the biosynthesis of different cellular components such as Carbohydrates, Lipids, Proteins, Nucleic acids… *(Soong et al., 2019).* *Rhodotorula sp* ecology and biodiversity cover a board of environmental varieties using a large variety of carbon resources, including Glycerol, Glucose, Sucrose, Galactose and Maltose, often encountered as dominant in yeast microflora (water, soil, vegetal and animals) *(Grigore et al., 2023).*

b. Nitrogen source

Nitrogen is one of the main parameters influencing the metabolic pathways of microorganisms and enzyme production *(Antonio et al., 2020).* It is important for protease growth and production *(Chellapandian et al., 2021).*

All yeasts are able to use nitrogen in the form of ammonium ion (chloride, nitrate, phosphate, but especially sulfate which is the ideal compound since it also provides sulfur necessary for the synthesis of certain amino acids). Some yeasts are able assimilate nitrites and nitrates as the only source of nitrogen *(Kurtzman et al., 2011).*

Nitrogen is generally supplied in the form of organic nitrogen compounds: peptone, yeast extract, corn steep liquor, etc. Yeast extract appears as the major element promoting the growth of yeasts, as it provides many amino acids and other forms organic nitrogen *(Deak, 2006).*

- ***The carbon/nitrogen (C/N) ratio***

These yeasts can biosynthesize both lipid and carotenoid. A few analysts optimized the carbon/nitrogen (C/N) ratio which gotten the most noteworthy surrender of the lipid and carotenoid. Depending on the species of *Rhodotorula*, the ideal C/N proportion has been diverse *(Hien and Deo, 2018).*

c. Trace elements and vitamins

Vitamins (Biotin, Thiamine and Inositol) can be building pieces of different coenzymes or prosthetic bunches of chemicals fundamental for the development of yeasts *(Kurtzman et al., 2011).* Other variables such as metal particles play a part imperative in advancement as they act as enzymatic cofactors and have moreover major auxiliary parts *(Normando et al., 2021).* Among antacid metals fundamental are the monovalent cations: K+, Na+, Li+, Cs+, Rb+, a

well as cations bivalents: Mg2+, Co2+, Zn2+ and Mn2+. Ca^{2+} particles have a part within the functioning of microfilaments, within the action of proteins and within the support of the basic judgment of layers *(Leveau and Bouix, 1993).*

d. Temperature

The ideal temperature for development is between 18 and 22°C. In vitro, the development is diminished at temperatures >33°C. The organism produces unicellular blastoconidia but does not produce pseudohyphae or indeed hyphae. Appearancies of different colonies of *Rhodotorula sp (Grigore et al., 2023).*

e. pH

PH moreover has an impact on yeast improvement which tend to colonize acidic situations and by their metabolic exercises acidifying the environment indeed more. Yeasts endure a wide pH extend from 2.4 to 8.6. Their ideal development happens at pH extending from 4 to 6.5 *(Chniti, 2017).*

f. Breathing and fermentation

It is vital to note that these forms are basically settled by natural components (*Ata et al., 2018).* All yeasts are able to create within the nearness of oxygen strict oxygen consuming *(Moreira,2019).* Others are aerobic-anaerobic discretionary favoring a digestion system either fermentative or respiratory (syn. *Pichia pastoris*) *(Ata et al., 2018).*

g. Osmotic pressure and water activity (Aw)

Varies from strain to strain. Most strains cannot be create water exercises underneath 0.90. A few water movement of the arrange of 0.60 but with a moderate digestion system. These yeasts are called xerotolerant, since they are able to synthesize osmoprotectors (betaine and glycerol) (**Table 02**) *(Houdaya, 2015; Dali and Hamame, 2016).*

Table02: Values of water activity of different micro-organisms

(www.rotronic.com).

water activity	Organisms
• **aw = 0.91 …0.95**	• Many bacteria
• **aw = 0.88**	• Many yeasts
• **aw = 0.80**	• Many halophiles
• **aw = 0.75**	• Halophilic bacteria
• **aw = 0.70**	• Osmiophilic yeasts
• **aw = 0.65**	• Xerophilic mold

6. Applications of yeasts in biotechnology

By their diverse metabolism, yeasts occupy an essential place in the industry agri-food *(Camara, 2018)*. They participate in the development of many products food (Bakery, Cheese, Brewery) *(Tsegaye et al., 2018)* and in production enzymes: Cellulase, β-Glucosidase, Protease, Lipase and Amylases *(Pretscher et al., 2018)*, from glycerol as well as vitamins and solvents (**Table 03**), but also to the upgrading of waste agricultural, industrial and protein production *(Houdaya, 2015; Ronnie, 2018)*. The Biotechnology and biomedical research also make extensive use of these microorganisms to the production of molecules of medical interest (e.g., production of heterologous proteins, such as hepatitis B vaccine) (*Camara, 2018; Liszkowska and Berlowska, 2021).*

Table 03: Some examples of industrial yeast applications Industrial applications Strains reference.

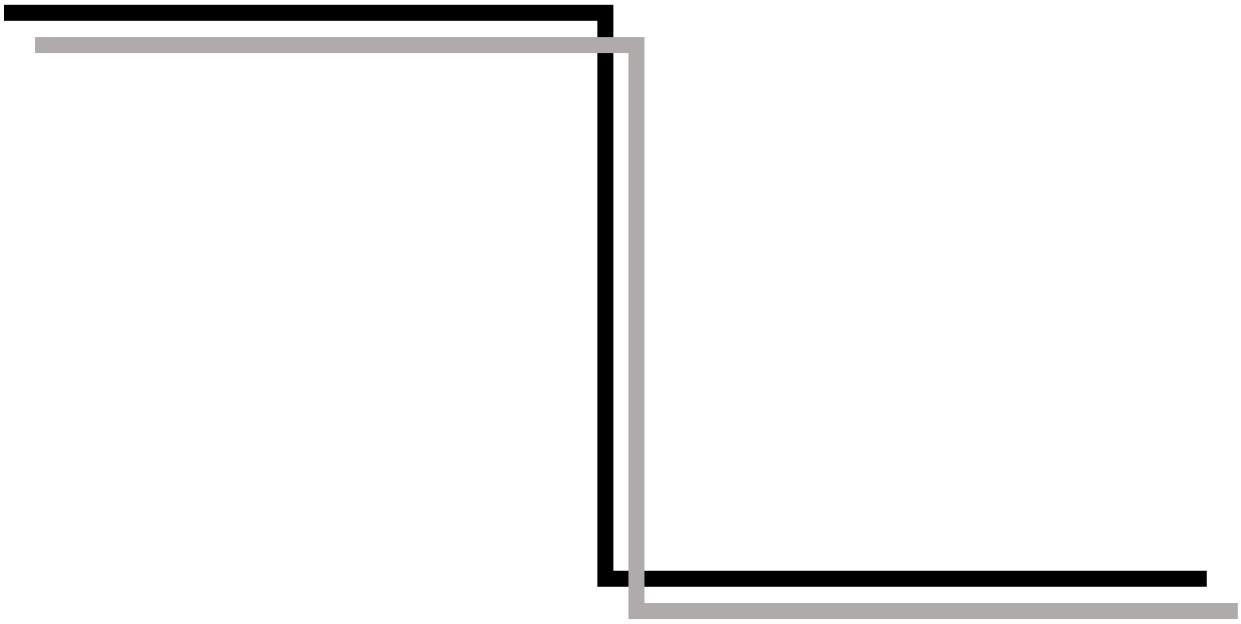
Applications industriels	Souches	Références
Fermentation alcoolique		
Vin		
Bière	*Saccharomyces cerevisiae*	Liszkowska et Berlowska (2021)
Alcool	*Brettanomyces bruxellensis*	
Cidre		Maicas (2020)
Fermentations sans alcool	*Saccharomyces pastorianus*	
Pain	*Torulaspora delbrueckii*	
Café	*Pichia anomala*	
Chocolat…		
Pharmaceutrique		Chniti (2015)
Antibiotique	*Streptomyces rimosus*	Villena *et al*., (2017)
Probiotiques	*Saccharomyces cerevisiae*	Branduardi *et al.*, (2008)
	Var. boulardii	
Hormones	*C. albican*	
Anti-inflammatories		
Hepatitis B vaccine	*Kluyveromyces lactis*	
Vitamine B 12…	*Kluyveromyces marxianus*	
	Metschnikowia gruessii	
Alimentation		Liszkowska et Berlowska (2021)
Protéines		
Production de fromages…	*Saccharomyces cerevisiae*	Meziane et Agagna (2019)
Additfs alimentaire	*Saccharomyces pastorianus*	
Arômes		
Pigments…		

Yeasts are also involved in the production of enzymes, and some examples are presented in (**Table 04**).

Table 04: Some enzymes produced by yeasts.

Enzymes	Souches	Références
Protéase	*Candida sake* *Saccharomyces cerevisiae* *D. vanrijiae,* *Cryptococcus taiwanensis* *Meyerozyma guilliermondii P.* *flavescens,* *P. laurentii* *R. mucilaginosa*	Maturano *et al*., (2015) Jaibon *et al*., (2016) Villena *et al*., (2017)
β – Fructofuranosidase	*Candida guilliermondii*	Bousmaha *et al*., (2007)
Lipase	*Yarrowia lipolytica*	Ya-Hue Soong *et al*., (2019)
Pullulanase **Amylopullulanase**	*Pachia pattoris* *Clavispora lusitaniae*	Akassou, (2018) Dakhmouche (2016)
Pectinase	*Candida sake* *Debaryomyces vanrijiae* *S. cerevisiae* *Candida sp* *Debaryomyces sp* *K. marxianus* *Pichia sp* *S. fragilis,* *Zygosaccharomyces sp*	Maturano *et al*., (2015) Gummadi *et al*., (2009) Villena *et al*., (2017)
Cellulase	*Saccharomyces cerevisiae*	Djeddou *et al*., (2018)
α-amylase	*Lipomyces kononenkoae*	Wanderly *et al*., (2004)
Glucoamylase	*Candida guilliermondii*	Lagzoli, (2007)
Hémicellulase	*Saccharomyces cerevisiae*	Djeddou *et al*., (2018)
Glucanase	*Hanseniaspora* *Zygosaccharomyce* *Komagataella phaffii*	Chessa *et al*., (2017)
β - galactosidase	*Candida kefyr* *Kluyveromyces sp*	Douchet *et al*., (2007) Lyutova *et al*., (2021)

Chapter 02



...Proteases...

1. Enzyme generalities

Enzymes are biomolecules that consist of amino acid subunits linked together by amide bonds. These biocatalysts have a high capacity to accelerate the rate and specificity of biological reactions by reducing the activation energy without any structural modification (*Adetunji et al., 2023)*. The active site of these macromolecules is housed in hydrophobic pockets, which determines their specificity for substrates *(Singh and al., 2016)* (**Figure 07**). Enzymes are secreted by living organisms and are required to sustain life. Enzymes play a crucial role in numerous biotechnological applications *(Prakash et al., 2013).* Proteases are considered the leading enzyme because of their versatility in biotechnology *(Gurung et al., 2013; Kieliszek et al., 2021).*

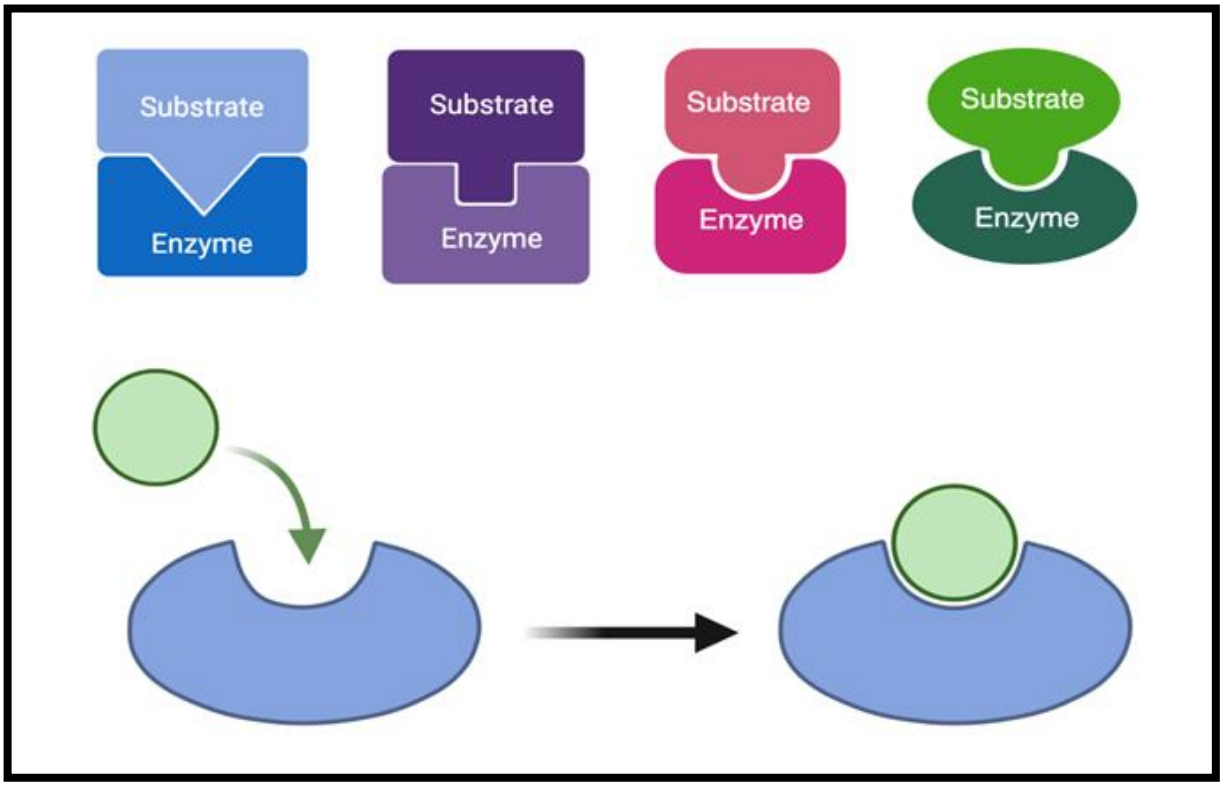


Figure 07: Structure of enzymatic active sites (*Dr. Saurav.Das, 2023*).

2. Enzyme market

The global industrial enzymes market was estimated to be valued at USD 6.6 billion in 2021 and to reach USD 9.1 billion by 2026 *(Research and Markets, 2022)*. 17.4% of the global enzyme market in 2024 will be oriented towards the manufacture of detergents. Carbohydrases accounted for 47.8% of the total enzyme market share, followed by proteases consumption 27.5% (**Figure 08**).

In 2024, a similar percentage distribution is expected, although the global enzyme market will increase from USD 8,184.2 million (2015) to USD 17,497.2 million (2024), an increase of 114% *(Grand View Research, 2018).*

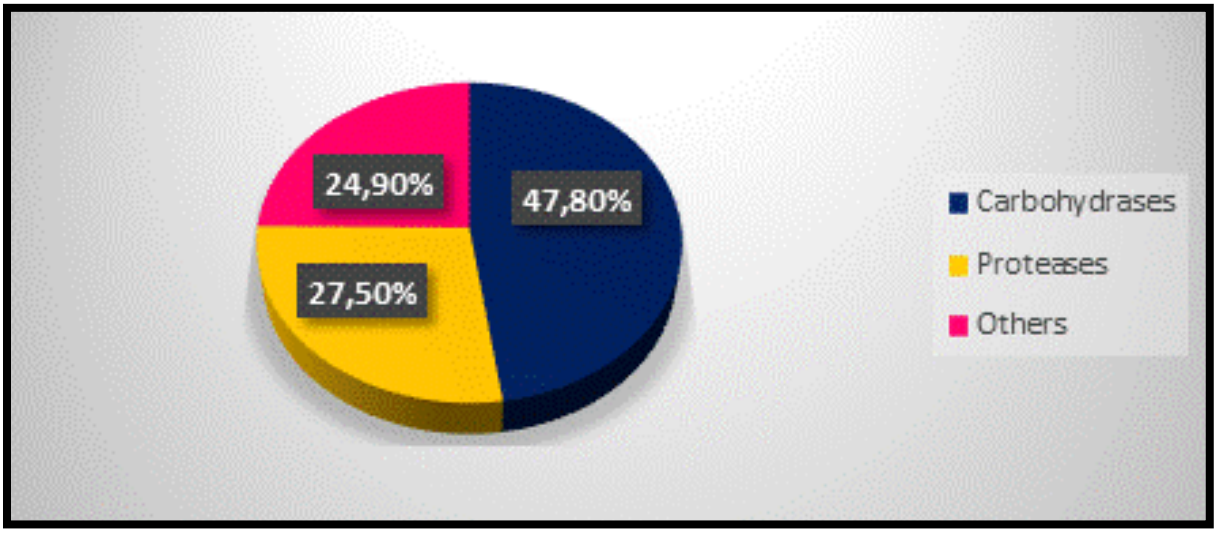


Figure 08: Enzyme market in 2024 *(Research and Markets, 2022.*

3. Proteolytic enzyme

Proteolytic enzymes are moreover known as proteinases, peptidases or proteolytic catalysts are a huge bunch of proteins having a put with the course hydrolases, related with preparing long protein chains into brief chains, cleaving the peptide bonds that interface amino corrosive buildups as illustrated in **Figure 09** *(Valmiki Aruna et al., 2023).* The main function is the splitting of proteins into smaller fragments *(Ravi et al., 2021).*

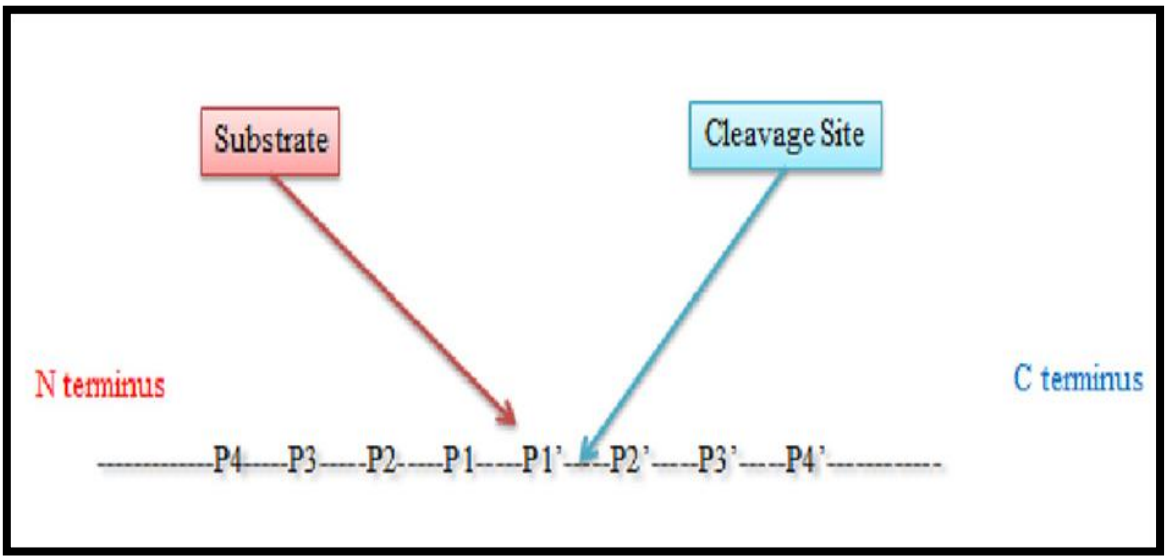


Figure 09: Action of protease on polypeptide chain *(Valmiki Aruna et al., 2023).*

Proteases are crucial in regulating various biological functions, including digestion, immune regulation, cell signaling, autophagy, differentiation, and modulation of the cell cycle, and they are essentially present in every organism *(Siqueira-Neto et al., 2018).*

Proteases are omnipresent, found in all living life forms and are fundamental for cell development, digestion system, signaling, and separation and other physiological purposes *(Banerjee et al., 2017).*

Most proteases are delivered as zymogen, the inert frame. Zymogens are enacted by natural signals or by assembly their particular substrate, this may result in a alter in adaptation (**Figure 10**) *(Dhillon et al., 2017).*

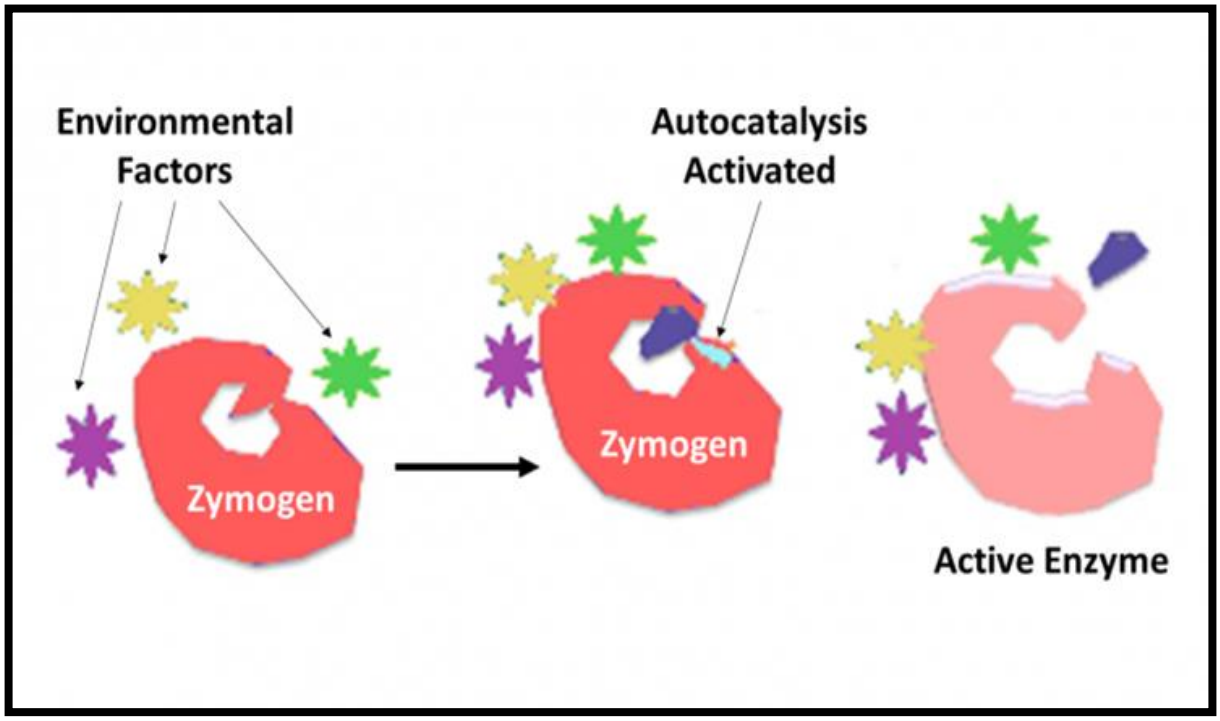


Figure 10: Activation of zymogen *(Stroud et al., 2019).*

4. The origins of proteases

4.1. Protease of plant origin

These plant proteases require the presence of the –SH- free group in their active site (cysteryprotease). The most known for their technological interest are papain, bromelain and keratinoases *(Sawant et al., 2014).*

Plant proteases are included in formskey physiological such as photo-inhibition, photomorphogenesis of seeds and senescence *(Dhillon et al., 2017).*

Papain, bromelain, cardosin, cynarases, and ficins represent some of the well-known plant proteases *(Bhunia et al., 2014).* Proteases of plant origin have applications in the food and food industries detergents *(Gaur et al., 2010).*

4.2. Protease of animal origin

The most excellent known proteases of creature beginning are trypsin, chymotrypsin, pepsin and renin. These are arranged in unadulterated frame in huge amounts. But, the generation of these proteins depends on animals' accessibility, which is administered by approach and breakage *(Mala, 1998).*

Chymotrypsin is found in animal pancreatic extricate; when it is immaculate, it is costly and is as it were utilized for symptomatic applications and expository *(Bhunia et al., 2014).*

Pepsin is one of the major proteins with noteworthy significance within the nourishment industry, biomedicines, and pharmaceutical details *(Ashaolu et al., 2023).* It is commercially

created from the gastric mucosa of bovine or porcine root, discharged in its zymogen frame (pepsinogen), and enacted beneath acidic conditions *(Nalinanon,2010).*

Renin may be a pepsin-like protease that's created as a precursor inactive within the stomach of all lactating well evolved creatures. It is converted to renin dynamic beneath the activity of pepsin. It is broadly utilized within the dairy industry to deliver a steady curd with great enhance *(Bhunia et al., 2014).*

4.3. Protease of microbial origin

Microorganisms, as source of proteases, offer a few inborn points of interest like quick generation rates, lower venture in terms of arrive and time necessities, not being subjected to climatic infuences *(Bhatia et al., 2021).* In addition, due to the different situations in which the microorganisms are found, it is very simpler to screenout the organisms creating chemicals with alluring characteristics *(Putatunda et al., 2019).*

Microbial proteases are among the foremost imperative and broadly examined hydrolytic chemicals since the starting of enzymology *(Gupta,.2002).* They constitute more than 40% of the whole around the world generation of enzymes *(Haddar et al,.2009; Raval et al., 2014).* They are delivered by a huge number of microbes, counting bacteria, fungi, and yeasts *(Kumar et al,.1999; Jisha et al,.2013).*

A) Bacteria

Bacterial proteases are the foremost imperative compared to creature proteases and contagious. Among the microbes, Bacillus species are the particular makers of extracellular proteases *(Das et Prasad, 2010*). A huge number of bacterial proteases has found, decontaminated and characterized, but exceptionally few of them are utilized in large-scale mechanical forms due to their tall fetched, trouble amid the decontamination and particularly the reality that these proteins are delicate in nature *(Kumar et al., 2008).* These proteases are neutral and are dynamic within the pH extend 5 to 8 and have a moo thermostability to bacterial antacid proteases *(Dhillon et al., 2017).*

B) Fungi

Like bacterial proteases, those from parasites are moreover broadly utilized. Mushrooms are flexible within the generation of chemicals and deliver acidic, impartial and soluble proteases, thus pH 4 and 11. Thermostability and the response rate of the contagious proteases isn't tall compared to those of microbes but the chemical delivered by the thermophilic organism *Malbranchea pulchella*. A critical number of *Aspergillus* species are known for their extracellular protease preparations *(Siala et al., 2009).*

C) Yeasts

A few yeasts moreover deliver proteolytic chemicals, it is basically of the genera *Saccharomyces, Rhodotorula, Candida, Debaryomyces*. *Saccharomyces cerevisiae* for case, produces three sorts of proteases; an aspartyl protease, a serine protease and a metallo-protease. The proteolytic movement of these genera is utilized especially for cheese maturing *(Kresze, 1991; Boiron, 1996).*

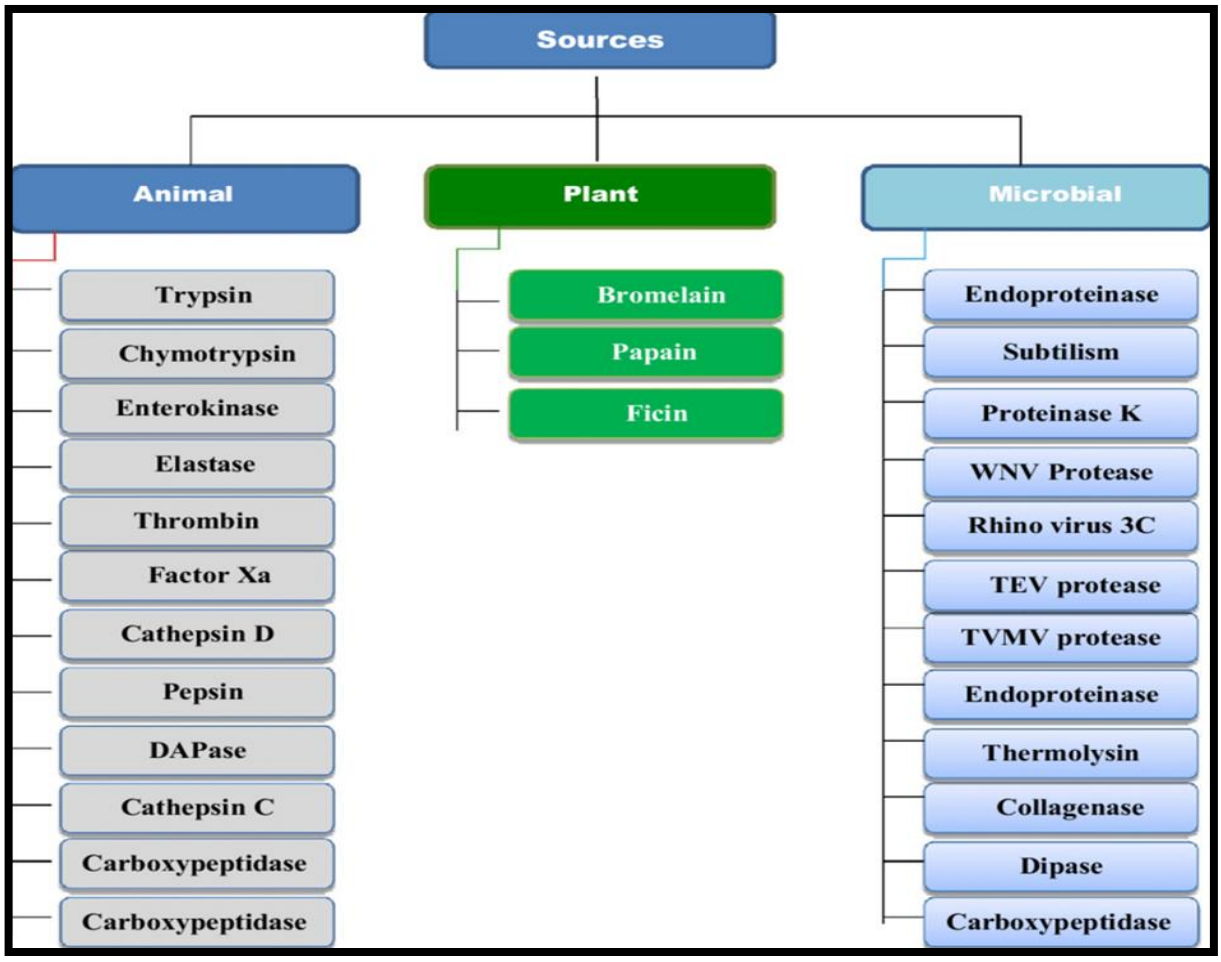


Figure 11: Proteases produced from different sources *(Muhammad et al., 2020).*

5. Classification of proteases

The classification of proteases is based on a few criteria such as the length of the polypeptide chain, chain assault mode, action pH and buildup nature included within the dynamic location *(Colwell and Grigorova, 1989; Rao et al., 1998).*

5.1. According to length of polypeptide chain

Typically the primary basis for the classification of proteolytic chemicals. There are two categories: proteases that part the protein particle into polypeptide parts and peptidases that hydrolyze polypeptides into free amino acids *(Frazier 1967; Colwell and Grigorova, 1989).*

5.2. According to the mode of attack of the polypeptide chain

Depending on their mode of assault, peptidases are subdivided into two classes: the endopeptidases and exopeptidases *(Scriban, 1999; Moodie, 2001).*

A) Exopeptidases

Act as it were close the closes of the polypeptide chains. A qualification is made between amino-peptidases acting on the N-terminal peptide bond and carboxypeptidase cleaving the C-terminal peptide bond *(Monteiro, 2015; Kiran, 2016)*:

- **Aminopeptidases:** (CE 3.4.14) act at a free N-terminal conclusion of the polypeptide chain and discharge a single amino corrosive buildup, dipeptide, or tri-peptide. (Monteiro and *al*., 2015).

- **Carboxypeptidases:** act at the C-terminal conclusion of the polypeptide chain and discharge a single amino corrosive or dipeptide. It can be separated into three major bunches, serine peptidases (EC 2.4.16), metallo-peptidases (EC 2.4.17), and cysteines peptidases (EC 2.4.18), based on the nature of amino corrosive buildups at the dynamic chemical location *(Monteiro, 2015).*

B) Endopeptidases

Are characterized by their particular activity at the level of peptide bonds within the inside districts of the polypeptide chain *(Monteiro, 2015).*

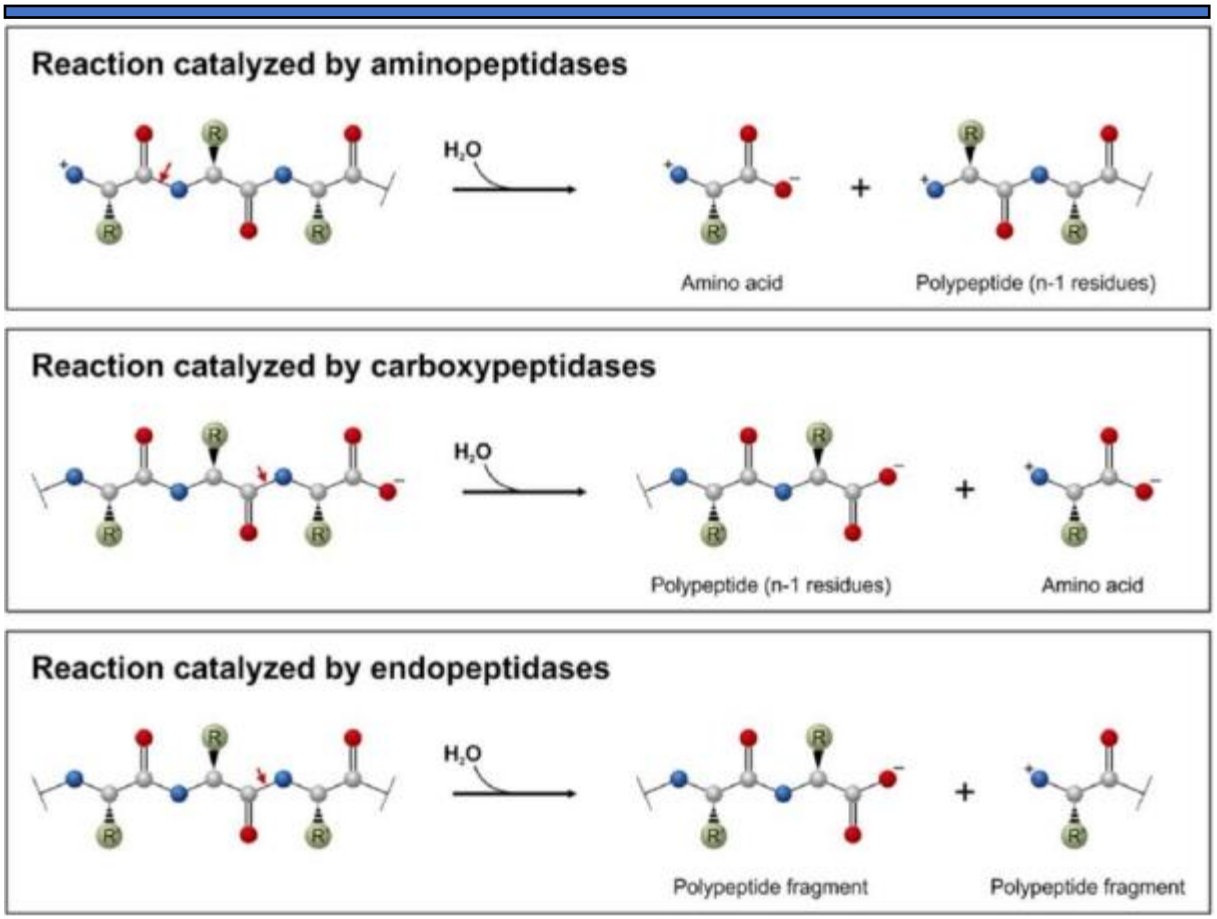


Figure 12: Action of aminopeptidases and carboxypeptidases removing acid residue terminal amino acids and endopeptidases on a polypeptide substrate. The arrows show that peptid bonds must be cleaved *(Mótyán et al., 2013).*

5.3. Classification by cell location

In this classification, we have the intracellular and extracellular proteases.

- **Intracellular protease:** Their primary work is the advancement and direction of forms cellular and metabolic, this kind of proteases is less utilized in businesses since these enzymes require a cell lysis step to form the extraction *(Mala et al., 1998).*
- **Extracellular protease:** The last mentioned catalyze the hydrolysis of proteins into little assimilable peptides. Their utilize in industries are curiously since they don't require cell lysis for extraction *(Mala et al., 1998).*

5.4. Depending on the type of residue involved in the active site

The fourth sort classifies proteases based on the nature of buildups included in the dynamic location. Hence, there are four bunches: serine proteases, cysteine proteases, aspartyl-proteases and metallo-proteases *(Mamo et Assefa; 2018).*

5.4.1. Serine proteases

Serine protease may be a clever basic hydrolytic chemical that utilizes its catalytic serine buildup for cleaving peptide bonds in proteins *(Zahoor et al., 2021).* Serine proteases are a course of multipurpose proteolytic chemicals. They are fundamental for the catabolism of proteins, the intracellular recharging of amino acids and the direction of proteins included in different atomic and cellular forms through taxa *(Rungaroon et al., 2021).* Serine protease constitutes a key component of the degradome of all life forms (**Figure 13**) *(Zahoor et al., 2021).*

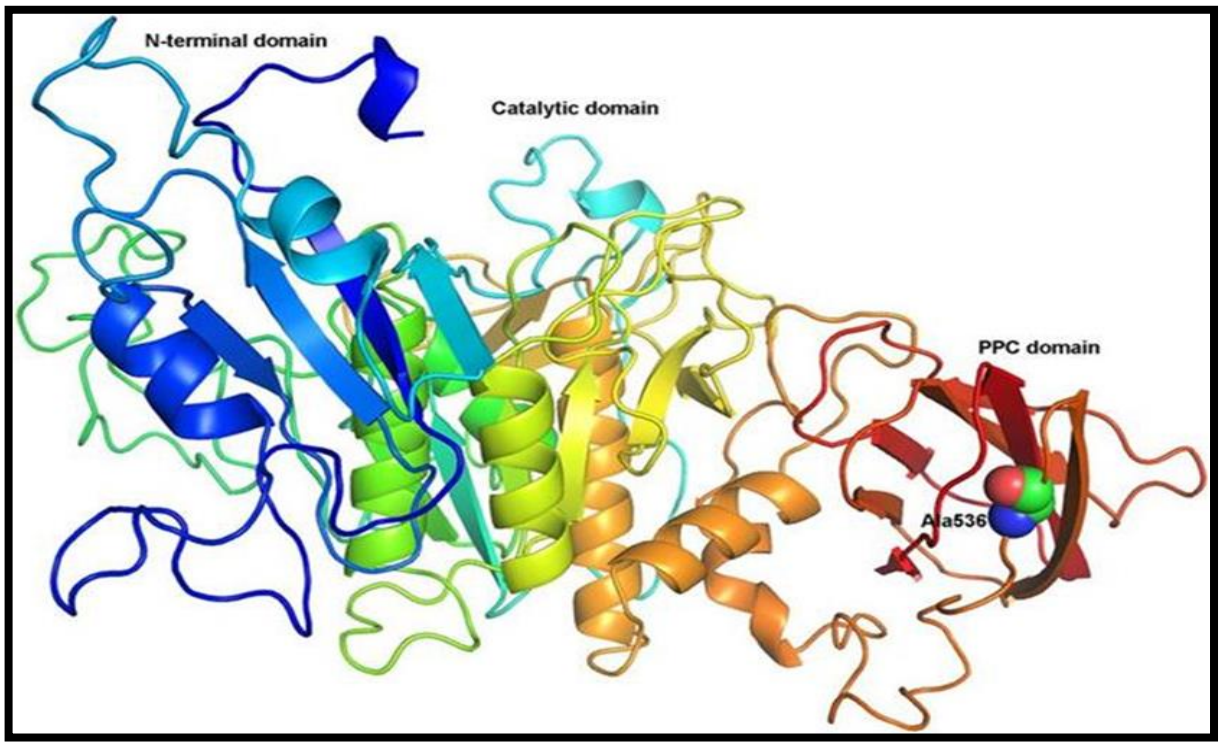


Figure 13: Serine protease structure *(Cheng et al., 2018).*

5.4.2. Cysteine proteases

Cysteine proteases catalyze the cleavage of protein peptide bonds, and speak to one of the four major bunches of hydrolases *(Grzonka et al., 2007, Buttle and Mort, 2013)*. These proteases are display in all microorganisms. As the numbers demonstrate, cysteine proteases are different and perform different capacities, from mass debasement to preserve homeostasis of proteins with exact cleavage to enact or age protein substrates (**Figure 14**) *(Sijwali et al., 2021).*

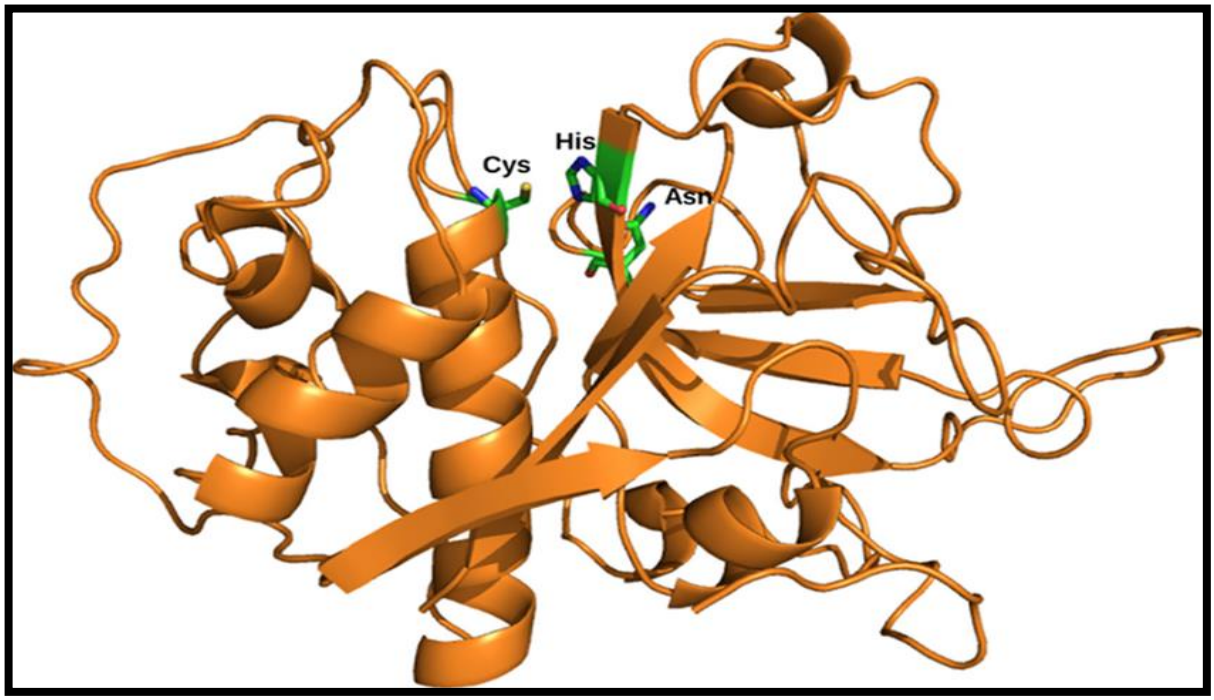


Figure 14: Cysteine protease structure *(Rajnikant et al.,.2016).*

5.4.3. Aspartyl proteases

Aspartyl proteinases are endopeptidases with two aspartic corrosive buildups (Asp32 and Asp215) in their dynamic location crucial for their catalytic action *(Yegin et al., 2011)* They are commonly alluded to as corrosive proteases *(Rao et al, 1998).* The last mentioned are classified into three families: Pepsin (A1), Retropepsin (A2) and Pararetrovirus proteins (A3). Most aspartic proteases (Aps) have the leading movement at moo pH (pH 3 at 4) and have isoelectric focuses over a pH run of 3 to 4.5 (**Figure 15**) *(Rao et al., 1998).*

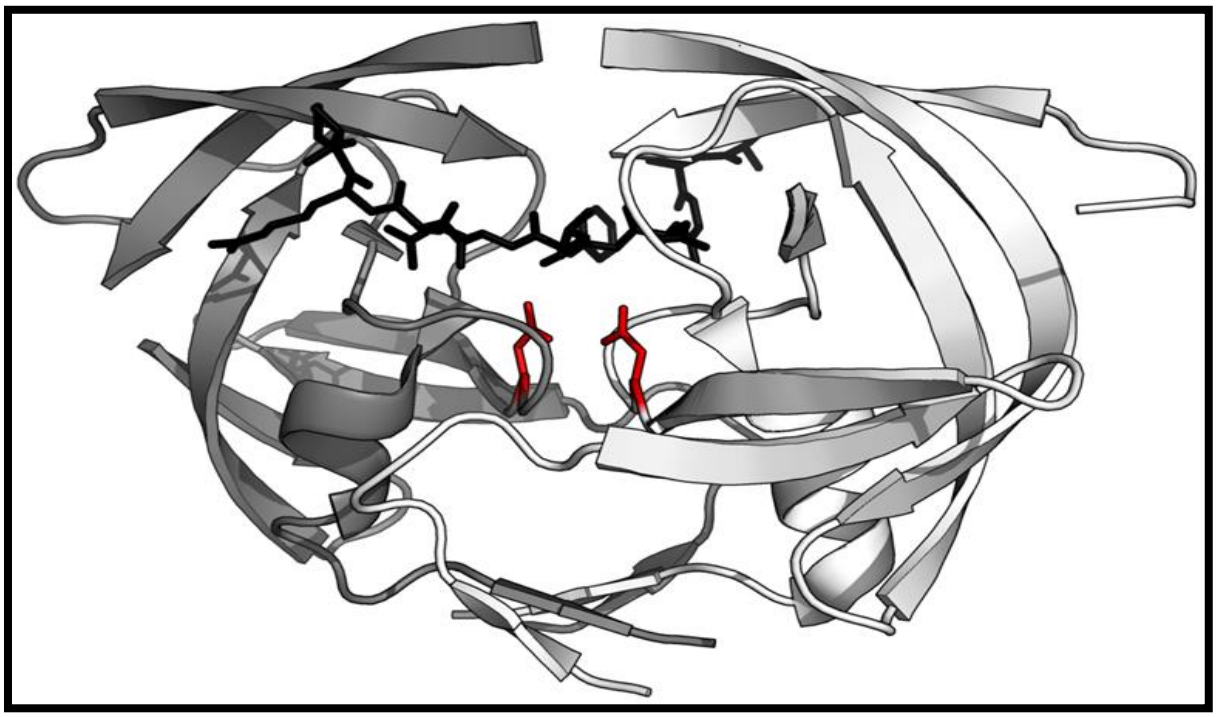


Figure 15: Structure of Aspartyl proteases *(Yegin et al., 2011).*

5.4.4. Metalloproteases

They are exceptionally different proteases. They contain chemicals of diverse beginnings, such as collagenases. For their activities, they require the foremost divalent metal particle frequently zinc, they require calcium for their solidness (**Figure 16**) *(Weron et Divol, 2014).*

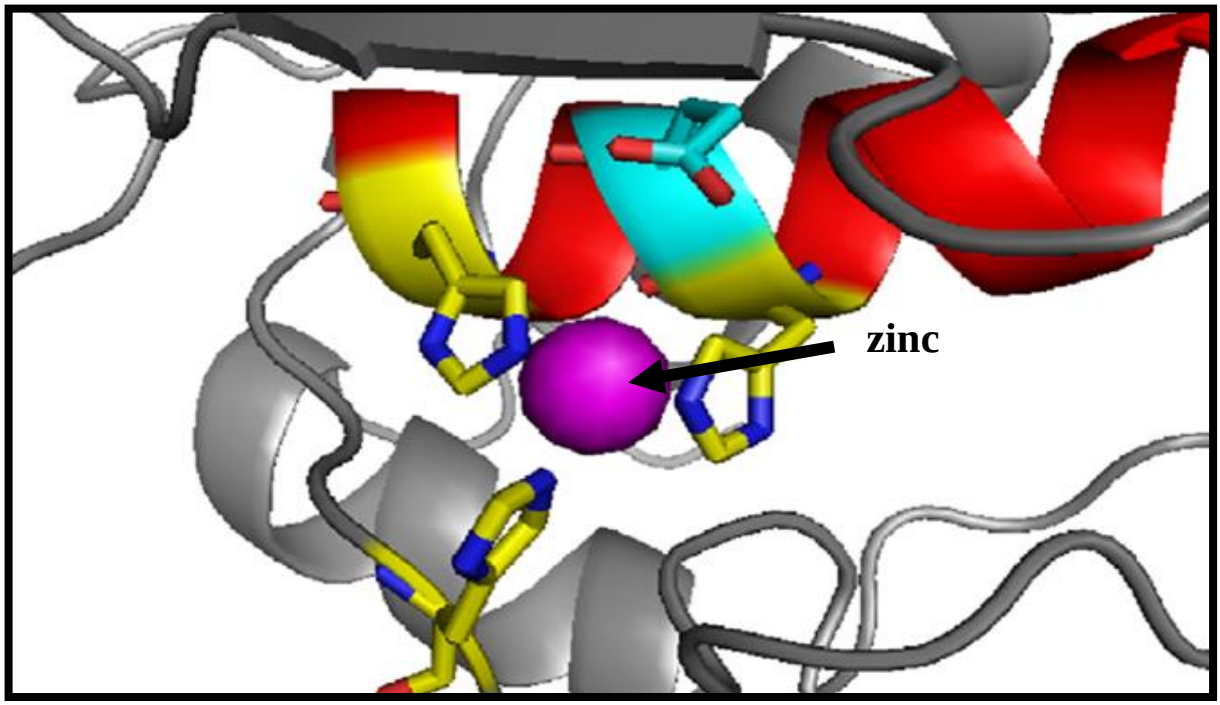


Figure 16: Structure of Metalloproteases *(David, 2007).*

5.5. According to the pH of activity

Proteolytic enzymes are classified according to the pH range in which their activity is optimal, in neutral protease (optimal pH 7), acid (optimal pH less than 7, between 2 and 5) and alkaline (optimal pH greater than 7) *(Sethia, 2016).*

Alkaline proteases produced from microorganisms play an important role Bacillus is an important source of these industrial proteases and it is probably the only genre marketed for their production.

Neutral proteases are mainly of plant origin with optimal activity at a pH between 8 and above, acid proteases are produced mainly by fungi and the best of these proteases are obtained in a pH range 2-5 *(Alnahdi, 2012).*

5.6. According to their need for ATP

This group of proteases incorporates those comprising of a few subunits containing ATPase and proteolytic spaces. There are proteases that don't have a place to any classification gather such as peptidal signals from lipoproteins *(Kumar et al., 1998).*

6. Mechanism of action of proteases

6.1. Serine proteases

The polypeptide is embedded into the serine protease in such a way that the carbonyl group is near to serine. The OH group of serine assaults the carbonyl group and histidine nitrogen acknowledges serine OH. A middle enzyme-substrate affiliation tetrahedral is shaped. At that point the chemical is acylated and the primary item of hydrolysis is discharged. Ina last response the protein is moved and the C-terminal conclusion is discharged (*Lopez, 2008*) (**Figure 17**).

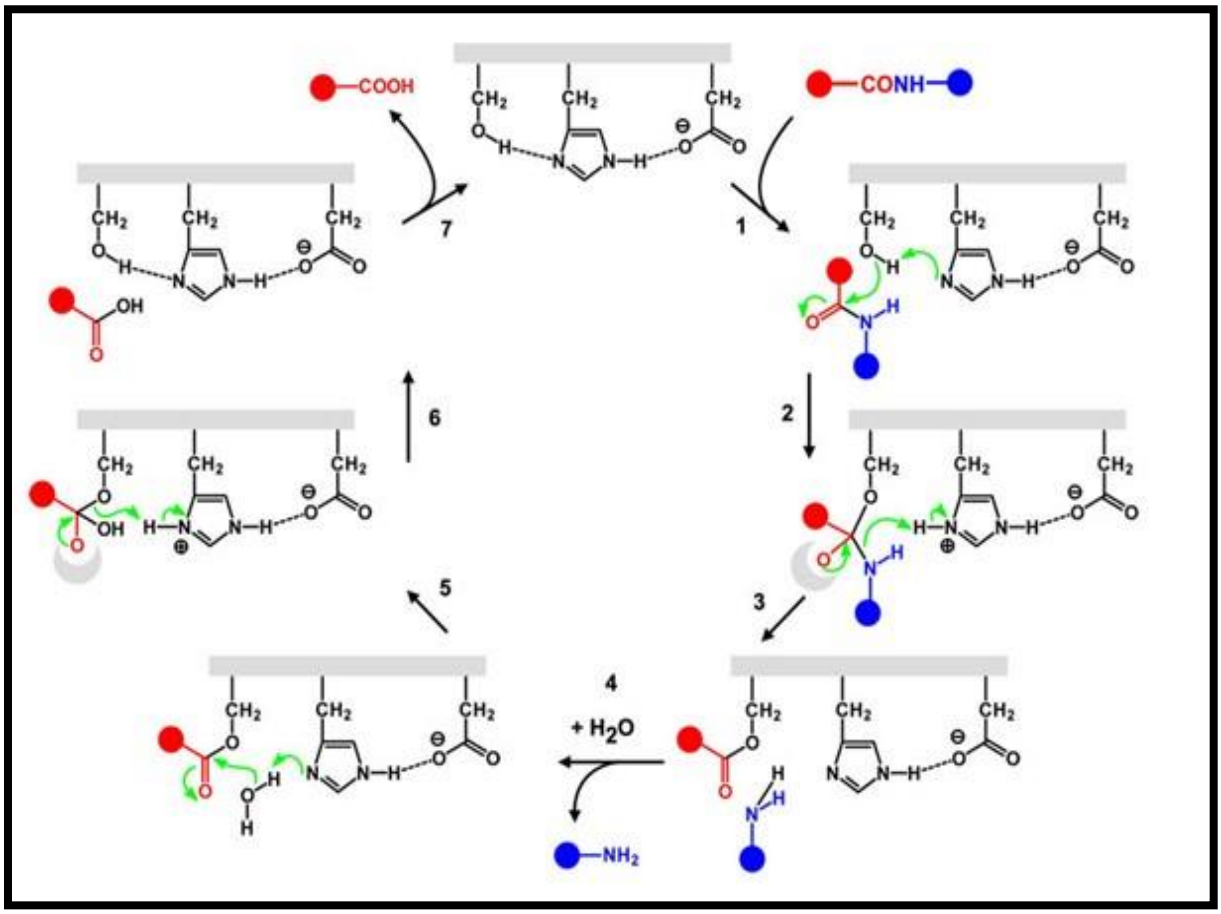


Figure 17: Serine proteases mechanism *(Rafal et al., 2017).*

6.2. Cysteine proteases

The primary step of the catalytic component of cysteinoproteases is the deproteinization of a thiol bunch within the dynamic location of the chemical, carried out by an adjoining amine acid with an essential horizontal chain (frequently it could be a histidine). The moment step comprises of a nucleophilic assault of the anionic sulfur of cysteine on the carbonyl gather of the substrate. In this step, a part of the substrate is discharged (**Figure 18**). Histidine within the protease is restablished in its deprotonized shape and a middle thioesther ties the carboxy terminal conclusion of the substrate to cysteine. The thioesther bond is hydrolyzed to produce a carboxy-terminal corrosive and the chemical is restablished *(Lopez, 2008).*

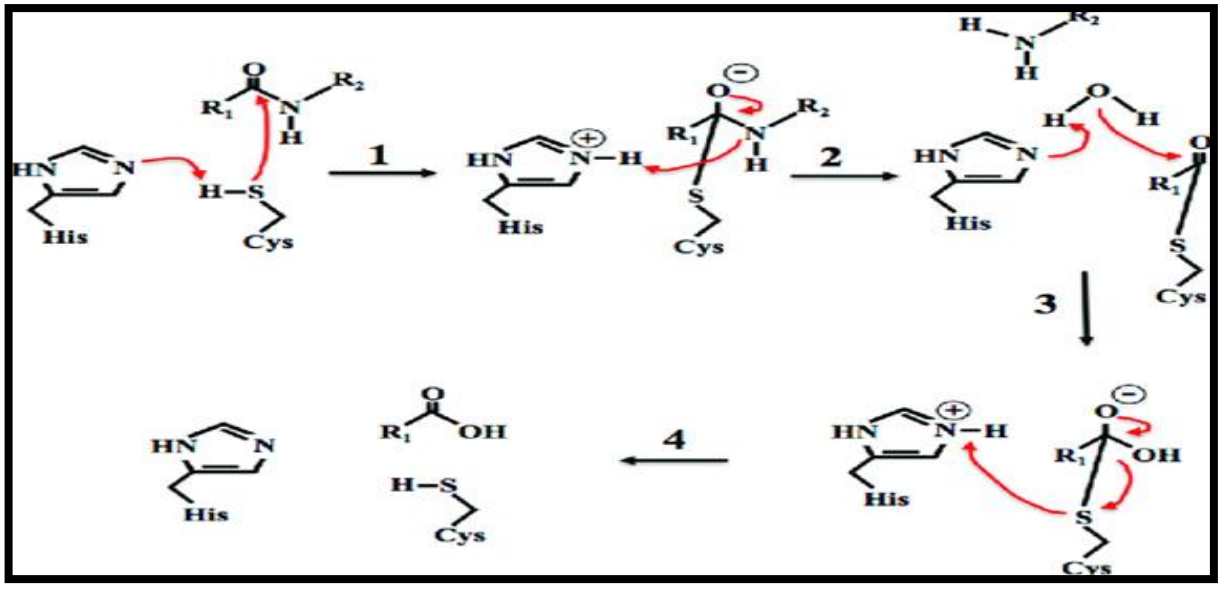


Figure 18: Cysteine proteases mechanism *(Allan Clay Clark, 2016).*

6.3. Aspartyl proteases

The component of activity of aspartyl proteases is as a rule acid-based, based on the coordination of a water particle between the two aspartates of the dynamic location **(Figure 19)**. An aspartate actuates the water particle by subtracting a proton. This enactment of the water particle permits an alteration of the carbonyl carbon of the substrate within the bond to be cleaved producing a middle of the road oxyanion. It is the improvement of this oxyanion that permits the cleavage *(Lopez, 2008).*

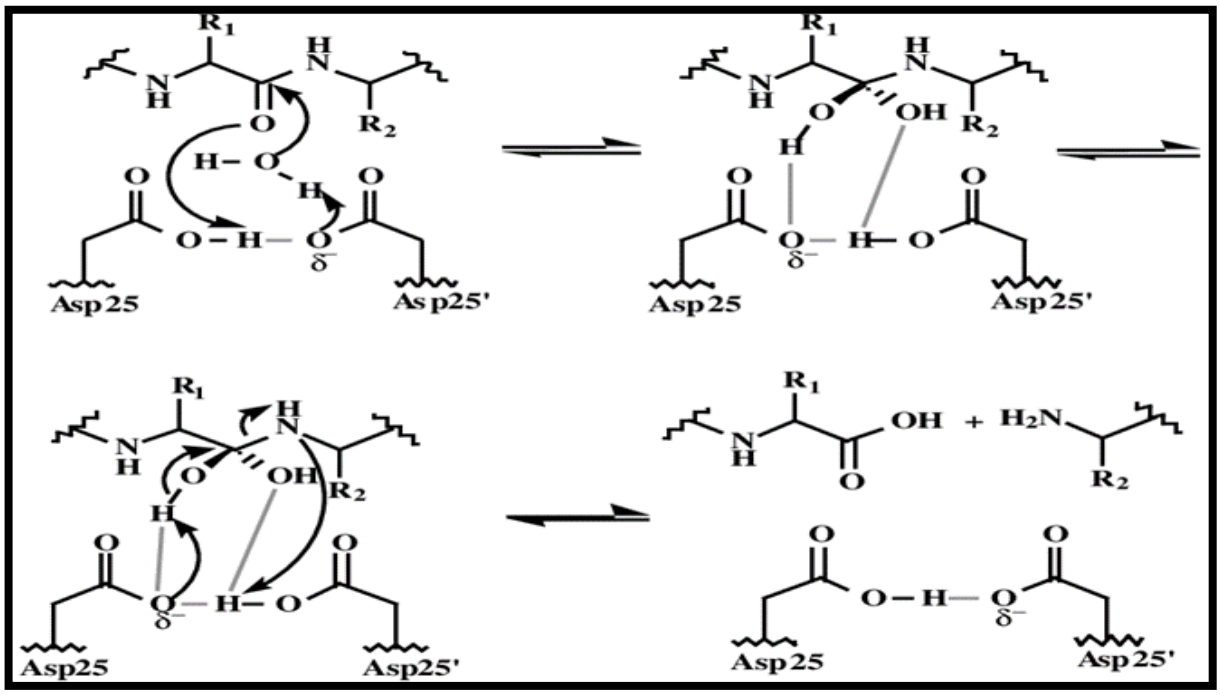


Figure 19: Aspartic protease mechanism *(Seddigh and Darabi, 2016).*

6.4. Metalloproteases

The component of hydrolysis commonly acknowledged is as takes after:

- ✓ The interaction of the water atom through its oxygen with the zinc iota actuates a polarization of this atom advancing the arrangement of a Gracious particle.

- ✓ The arrangement of this species is favored; from the proton of the water atom to the glutamate buildup, of which the carboxylate work of its side chain changes from COOP state within the COOH state. At the same time, the substrate to be hydrolysed was situated within the dynamic location so that the carbonyl of the scissile peptide bond can too connected with the zinc iota.

- ✓ The zinc molecule in this way sees its state of coordination from tetrahedral to pentacoordinated. This transitory structure permits the nucleophilic assault of the carbon of the gather carbonyl, polarized by its interaction with zinc, by the OHP particle (**Figure 20**).

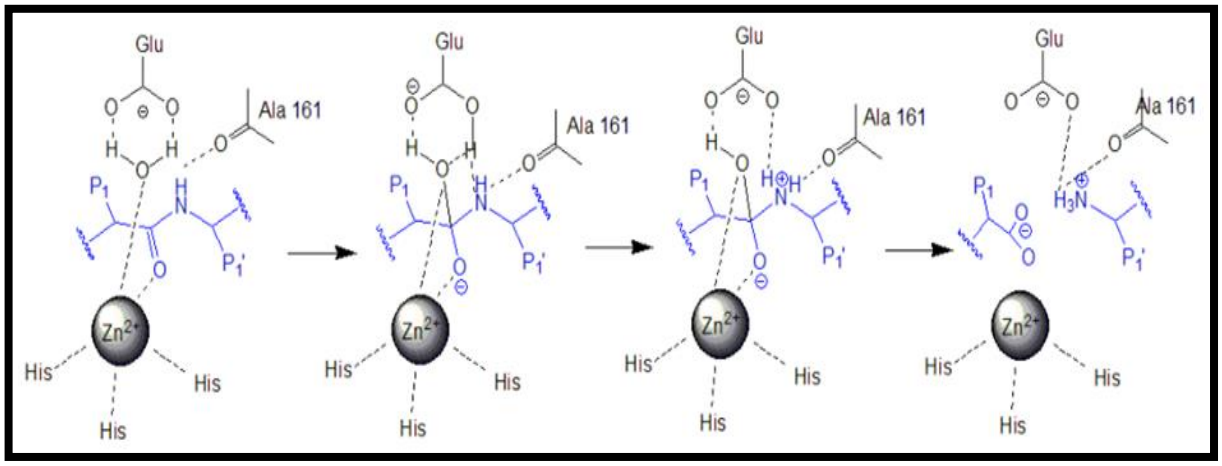


Figure20: Metalloprotease mechanism *(David, 2007).*

7. Protease applications

Table 05: Protease applications.

Applications	Principe
1.Agro-food industry	**1.Cheese factory:** Within the dairy industry, proteases a basically utilized within the fabricate of cheese to hydrolyze particular peptide bonds to create casein and macro-peptide *(Singh et al., 2016).* Proteases are included to the hydrolysis of kappa casein amid the generation of making cheese from drain, to anticipate coagulation whereas stabilizing the arrangement of micelle *(Abdul Razaaq et al., 2019).* **2.Baking:** In the baking industry, they are included to guarantee consistency of mixture, diminish the consistency of the mixture, keep up the strength of gluten within the bread and make strides the enhance and surface of bread. These hydrolytic chemicals are utilized to debase the turbidity complex coming about from proteins in natural product juices and alcohols liquor, in gelatin, hydrolysis and protein recuperation from meat *(Singh et al., 2016).* Within the pastry kitchen industry to quicken mixture arrangement its gluten is somewhat hydrolyzed by a thermolabile parasitic protease. Planning tall wholesome esteem protein hydrolysate was gotten by the expansion of proteases microbial *(Abdul Razaaq et al., 2019)*. **3.Soy products:** Neutral and alkaline proteases are used to prepare soy sauce The processing of soy proteins by alkaline protease to pH 8 allows the development of protein hydrolysates solubles that are used as protein additives in the food *(Lakba et Soucha, 2015).* **4.Synthesis of aspartame:** proteases they can sometimes catalyze the reverse reaction, sometimes a preparation of thermolysine is used for the synthesis of aspartam from aspartic acid and Lphenylalanine methyl ester It is produced industrially by Toya Soda (Japan) *(Leisola et al., 2001; Belmessikh, 2011; Zemmouri et Zemouri, 2017).*
2.Detergents industry	Chemicals are the critical component of powdered and fluid cleansers, evacuating clothing stains, cleansers for the programmed dishwasher,cleaning items . Proteases are one of the foremost vital fixings of all sorts of cleansers extending from family clothing items to reagents utilized for cleaning contact focal points or denature. Proteins progress the viability of cleansers for protein evacuation of dress dirtied with sweat, grass, drain, egg, blood, etc *(Patel et al., 2018).*
3. Wast management	Proteases are utilized to handle different shapes of squander,counting strong, fluid, and unsafe squander. Protease proteins too help in squander debasement by changing over squander little straightforward particles which will use by other living beings for metabolic exercises and hence offer assistance lower the organic oxygen request of

	sea- going frameworks. A detailing containing hydrolytic chemicals inferred from B. *subtilis*, B. amyloliquefaciens, and Streptomyces *sp*. has been arranged and licensed as Genex for depilation and cleaning of hairs from channels and clogged channels *(Naveed et al. 2021, Estate et al ,2013)* detailed the application potential of keratin hydrolysates created from quills for detailing of hair shampoos. A thermostable and pH-stable protease from Micromonospora chaiyaphumensis S103 illustrated great potential for deproteinization of shrimp squanders for chitin handling *(Mhamdi et al. 2017).*
4.Leather industry	The utilize of proteases as an elective to unsafe chemicals such as sulfide of sodium has been appeared to be compelling in progressing calfskin quality and decreasing the environment. Tanneries are capable for natural contamination. By in this manner, the utilize of protease-like hydrolysis chemicals in calfskin handling could be an ecologically neighborly prepare that's less destructive than other forms *(Kumar et al., 2018; Singh et Bajaj, 2017).*
5.Brewing industry	In brewing, microbial proteases have two fundamental applications. They can be utilized amid the cereal brewing prepare to extend the abdicate of the amino nitrogen substance of the wart created. The protease is utilized within the generation stages of brew, in spite of the fact that papain, bromelain and pepsin are the proteases utilized in cold prepare security . Streptomyces fradiae proteases are utilized with victory for lager clarification *(Ami et al., 2019).*
6. Medical industry	It has been detailed that clostridial collagenase or subtilisin have been utilized to within the arrangement of a few anti-microbials, whereas asparginase, a protease that dispenses with aspargine, delivered by E. coli is successful within the treatment of lymphocytic leukemia. Proteases too act as anti-inflammatory specialists. The bacterium Serratia Sp. has created an extraordinary protease called serratio-pepetidase, which is able to diminish irritation rapidly. It has been expressed that genetically adjusted microbes are exceptionally successful totally different sorts of cancer treatments, counting focused on hindrance of cell signaling by bacterial protease, such as streptokinase generation *(Banerjee et Beam, 2017).*
7.Photographic industries	Silver is one of the valuable and respectable metals utilized in huge amounts in numerous purposes, particularly within the photographic industry. Film squander radiographic/photographic containing dark metallic silver spread in gelatin are a really great source of silver recuperation compared to other sorts of movies. The diverse conventional strategies of silver recuperation are: combustion coordinate from the movies, oxidation of metallic silver after electrolysis, stripping of the silver

	gelatin layer with utilize of distinctive chemical arrangements. But this strategy has genuine natural impediments *(Chander, 2019).* An acid proteases delivered by B. *subtilis*, Streptomyces avermectnus, and Conidiobolus coronatus were effectively detailed to recoup cash from motion pictures radiographs, which guarantees that this handle is more ecologically neighborly than the utilize of chemicals. The recuperation of silver by the viable utilize of a mutant soluble protease thermostable delivered by Bacillus *Sp*. B21-2 has too been detailed for its potential (Razzaq and *al*., 2019).
8. Bioremediation	The utilize of chemicals in businesses is inconvenient to the environment. This unsafe utilize of chemicals calls for a arrangement elective and ecologically neighborly squander treatment and administration. Poultry plumes contain a really inflexible keratin structure, speak to 5% of the body weight and are a wealthy source of protein for nourishment and bolster *(Chander, 2019).* Poultry squander can be debased into nourish and nourishment nourishment through the keratiniolytic handle *(Chander, 2019).* For hair evacuation and cleaning hair from blocked channels and channels, a detailing containing proteins hydrolytic disconnected from B. *subtilis*, B. *amyloliquefaciens* and *Streptomyces Sp*. arranged and licensed as Genex *(Razzaq et al., 2019).*
9.Other applications	Proteases from biological sources were randomly used inresearch purposes such as the alkaline protease produced by C. *coronatus* that replaces the trypsin in animal cell cultures *(Banerjee et Ray, 2017).* In addition to their industrial and medicinal applications, proteases play a role important in basic research. They are also used for the cleavage of peptide binding to elucidate the link between the structure and function of peptides and proteins. The alkaline proteases isolated from Vibrio metschnikovii RH530 can be used as an alternative to proteinase K in DNA isolation *(Razzaq et al., 2019).*

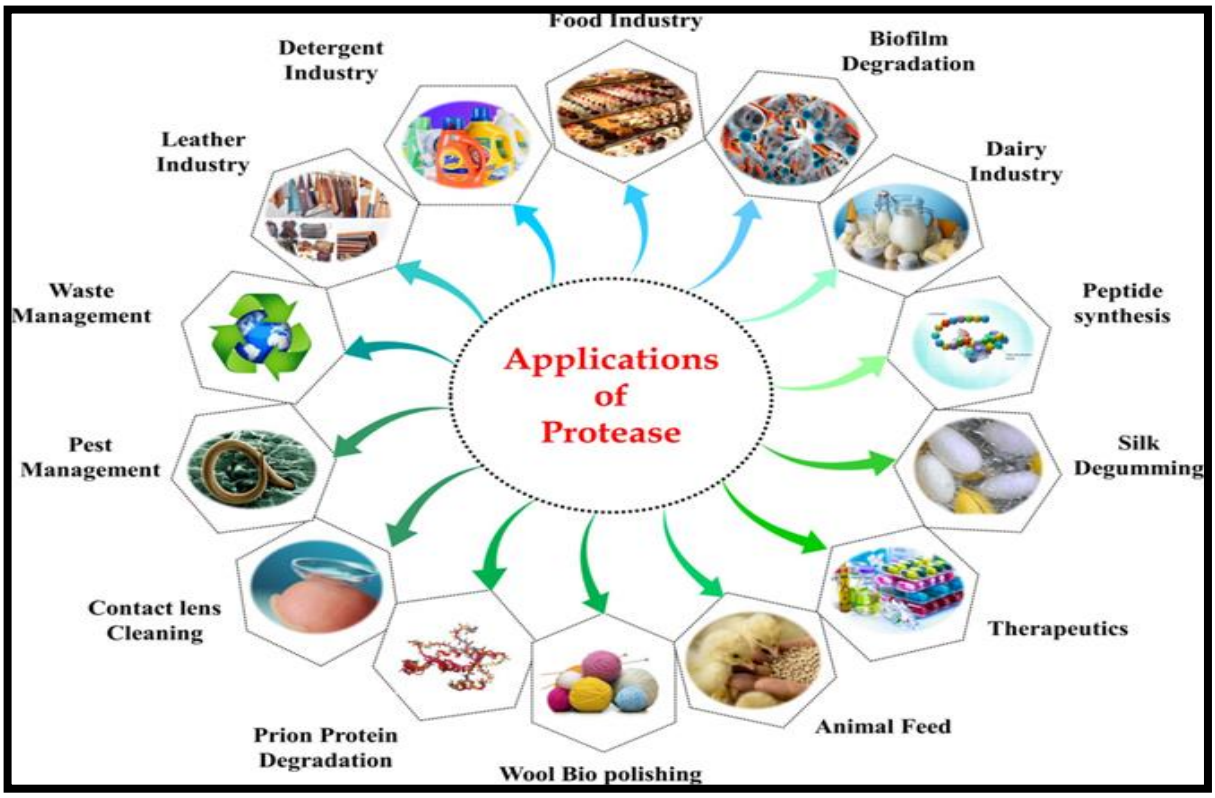


Figure 21: Applications of protease enzyme in various industries

(Solanki et al,.2021).

8. Factors influencing enzyme activity

8.1. Temperature

An increment in temperature increments the speed of development of atoms that will subsequently increment the number of collisions between substrates and enzymes, warm impedes enzymatic action by breaking the bonds between amino acids of the chemical such as hydrogen bridges and ionic bonds that keep up the compliance of the enzyme.

A decrease in temperature moderates down atomic disturbance and, thus,the plausibility of assembly between the enzyme and its substrate, The enzymatic speed diminishes, since proteins seldom tie to the substrate. Proteins have a temperature for which they are most viable *(Bluteau et al., 2021).*

Not all enzymes have the same ideal temperature, but most of them they act within the human body at an ideal temperature of 37.5°C *(Nagwa, 2022).*

8.2. pH

Depending on the pH of the medium, the emphatically charged cationic frame, or the

adversely charged anionic frame of amino acids will be advanced. This impacts intelligent between amino acids of inverse charge which can lead to changes within the form of the chemical within the tertiary and quaternary structure. The enzyme is not able to oblige the substrate at the dynamic location. In this way, a lower pH or higher than ideal leads to lower protein action. *(Bluteau et al., 2021).*

In spite of the fact that numerous enzymes within the human body have an ideal pH of 7, Not all enzymes have the same ideal pH *(Nagwa, 2022).*

8.3. Substrate and enzyme concentration

The increment in substrate concentration leads to an increment within the reaction rate until there's immersion.

Once all dynamic destinations of the enzyme are utilized, increment the concentration within the substrate will not have a positive impact on the response rate since has more dynamic free destinations for extra substrates, In this circumstance, expanded protein concentration would permit for more dynamic destinations accessible to suit substrate (**Figure 22**) *(Amiour and Bendjeddou, 2022).*

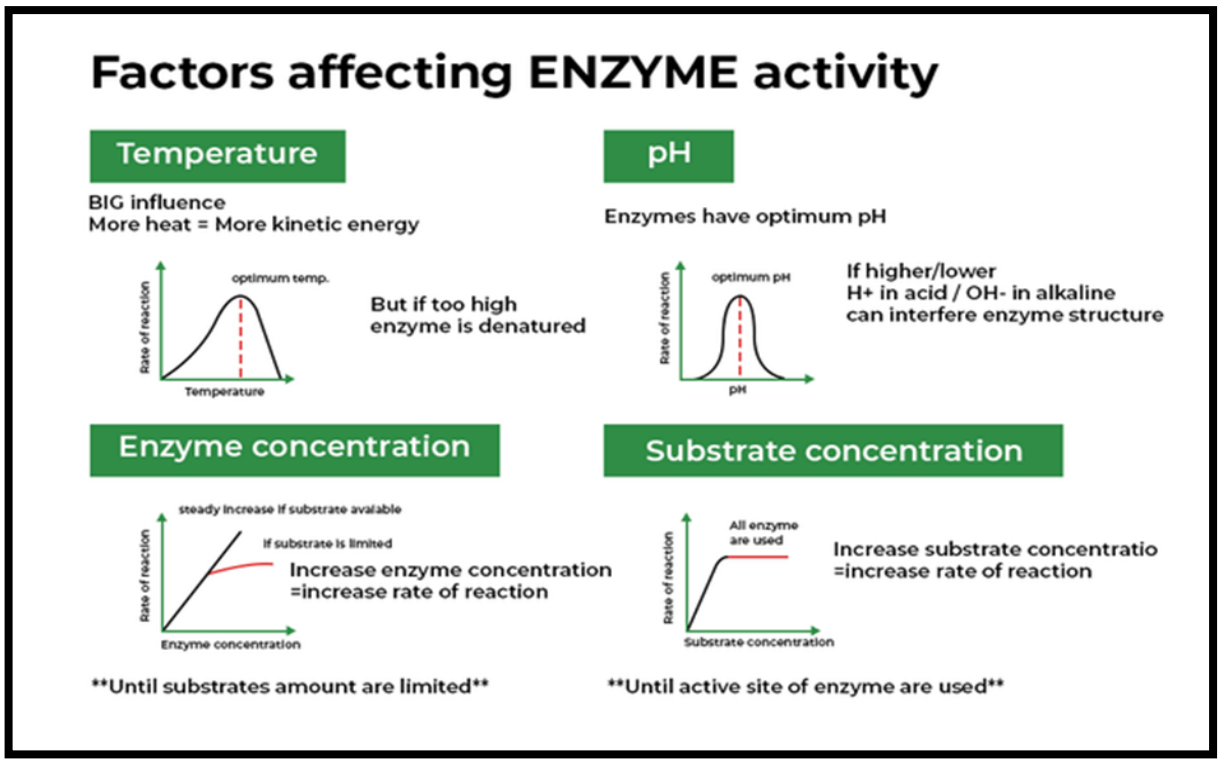


Figure22: Factors affecting Enzyme activity *(Amiour and Bendjeddou, 2022).*

8.4. Enzymatic inhibitors

There are competitive inhibitors that take a frame comparative to the substrate andsettles at the dynamic location. The chemical cannot total a response and action enzymatic is decreased. There are non-competitive inhibitors that misshape the dynamic location by being bound somewhere else on the protein, so the adequacy is less *(Bluteau et al,.2021).*

On the off chance that these inhibitory impacts happen on a huge scale on numerous chemicals included in a response, this may essentially diminish the speed of enzymatic responses. Subsequently, an increment within the concentration of enzymatic inhibitors will to diminish the response speed (**Figure 23**).

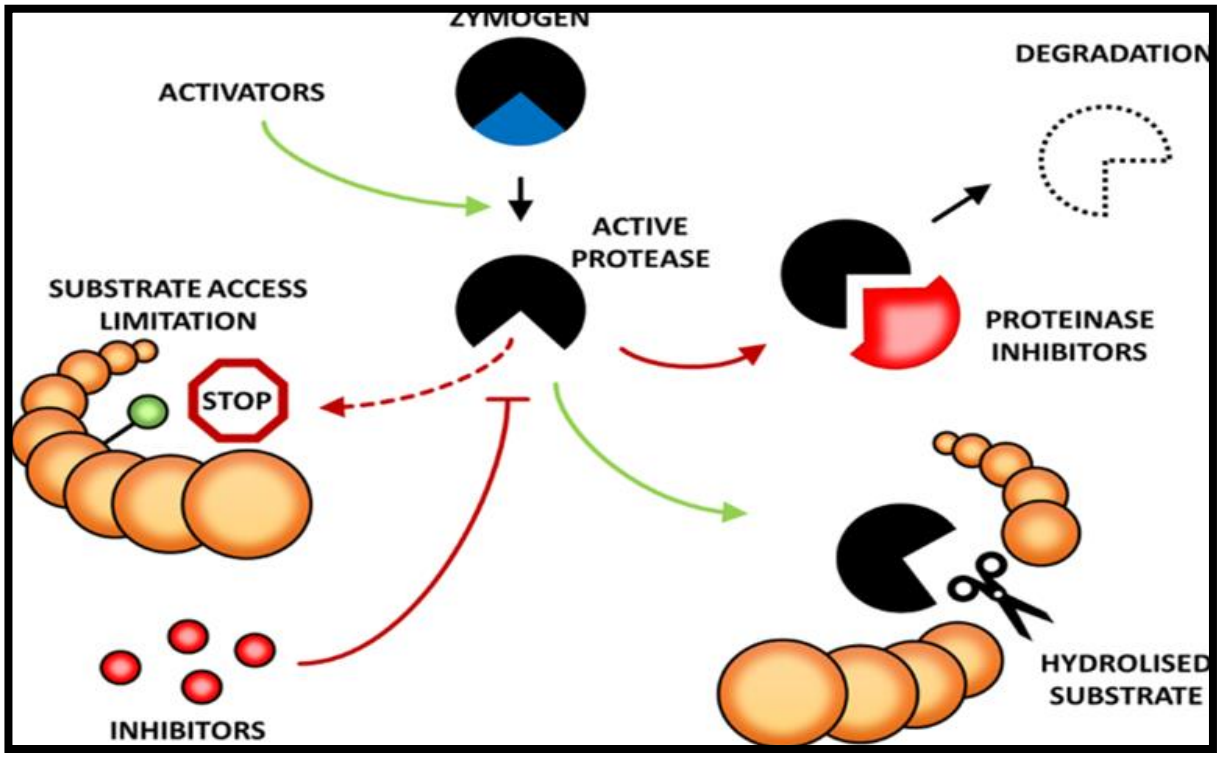


Figure 23: Activation and inhibition of enzymes (Daniel et *al*., 2023).

9.Recent approaches to improve protease yield

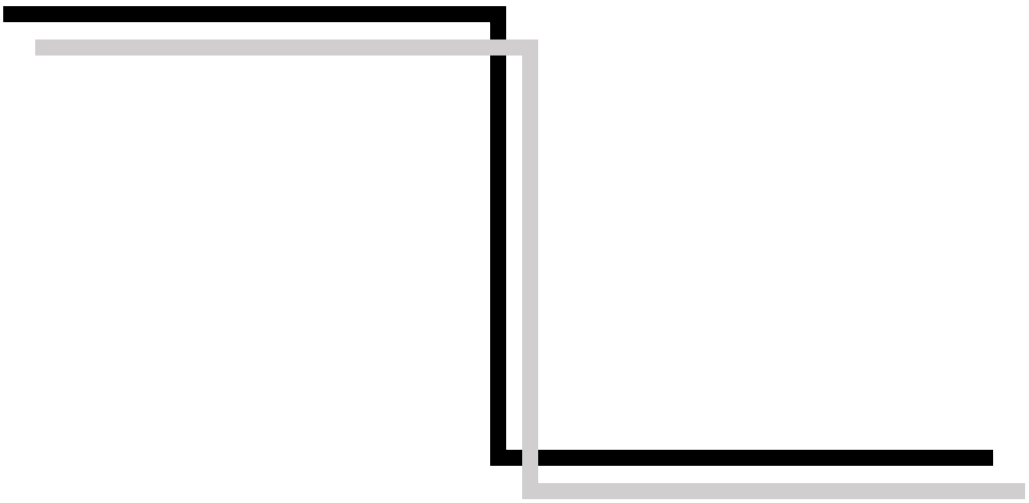
To improve yield of protease protein from microbial sources, researchers have utilized diferent methods counting cloning and overexpression, bolstered group and chemostat aging, and optimization of parameters (medium and development conditions) utilizing one figure at a time strategy and factual approaches such as reaction surface strategy. The nonstop eforts are being made to improve the generation of protease protein utilizing conventional i.e., UV or chemicals as well as later strategies i.e., hereditary building, quality altering by clustered routinely interspaced brief palindromic rehashes (CRISPR), protein

building and metagenomics *(Thakur et al. 2020)*. The focal points and drawbacks of thesemethods are summarized in **Table 05**.

Table 06: Selected novel microbial proteases and their potential application (*Solanki et al,.2021).*

Microorganism	Source of isolation	Type of protease	Potential application	References
Alcaligenes faecalis APCMST-MKW6	Marine sediment	Halophilic organic solvent tolerant protease	Deproteinization of shrimp shell waste	Maruthiah et al. (2016)
B. subtilis	Soil from tannery area	Alkaline protease	Animal hide dehairing, detergent additive	Hussain et al. (2017)
Halobacillus sp. CJ4	Hypersaline lake	Thermo-solvent stable protease	Peptide synthesis and detergent formulation	Daoud et al. (2017)
Aspergillus foetidus	Savannah soil	Acidic protease	Food industries	Souza et al. (2017)
B. subtilis AKAL7 and *Exiguobacterium indicum* AKAL11	Poultry waste mixed soil	Alkaline protease	Animal hide dehairing, removal of gelatin from X-ray film	Hakim et al. (2018)
B. halotolerans strain CT2	Tunisian potato	Alkaline protease	Detergent additive	Dorra et al. (2018)
Bacillus cereus FT 1	Organic matter mixed soil	Alkaline protease	Detergent additive	Asha and Palaniswamy (2018)
B. tequilensis	Soil	Fibrinolytic protease	Blood clot dissolution	Xin et al. (2018)
B. safensis strain RH12	Off-shore oil fields	Serine alkaline protease	Detergent additive	Rekik et al. (2019)
Salipaludibacillus agaradhaerens strain AK-R	Soda lake	Serine alkaline protease	Detergent additive	Ibrahim et al. (2019)
Lactobacillus paracasei 2.12	Goat milk	Rennin-like protease	Milk clotting activity	Putranto et al. (2020b)
Streptomyces sp. Al-Dhabi-82	Soil	Thermostable alkaline protease	Keratinous waste degradation	Al-Dhabi et al. (2020)
B. atrophaeus NIJ	Hydrocarbon-contaminated soil	Serine alkaline protease	Detergent additive	Rahem et al. (2021)
B. stearothermophilus	Olive oil mill sols	Thermostable alkaline protease	Detergent additive	Karray et al. (2021)

Chapter 03



..Fermentation..

3. Fermentation

3.1. Solid State Fermentation (SSF)

Solid State Fermentation may be a maturation prepare in which microorganisms develop on sodden, strong fabric beneath controlled conditions, without the nearness of free water or with a negligible sum of free water. Inactive or non-inert materials can be utilized as strong substrate in SSF forms *(Gordana Šelo et al., 2021).*

SSF can be characterized as a handle in which particles strong, with a ceaseless inter-particle gas stage, acting as a substrate, or as strong back for the development of microorganisms, within the nonappearance of open water *(López et al., 2020)*. It normally produces, for case, the deterioration of strong natural matter, FMS for the generation of nourishment and beverages such as within the case of bread, cheese, soy sauce or cocoa and its preparing of coffee *(López and al., 2021).*

3.1.1. Micro-organisms used in FMS

The microorganisms utilized in SSF are filamentous organisms, yeasts, and microbes. Due to their physiological, biochemical, and enzymatic properties, filamentous organisms (multicellular living beings) are the foremost commonly utilized, particularly those from parasitic kingdom subdivision Basidiomycota and Ascomycota *(Gordana Šelo et al., 2021).*

In arrange to create a dependable and repeatable SSF prepare, analysts ought to carry out this prepare in particular sorts of bioreactors beneath controlled conditions, such as plate bioreactors, turning plate reactors, settled bed bioreactors, column bioreactors, discuss weight throb strong state bioreactors, turning level drum bioreactors, blended drum bioreactors, fluidized bed bioreactors, air-lift bioreactors, and immersion bioreactors. The foremost reasonable sort of bioreactor for SSF scale-up is the plate bioreactor. It may be a conventional sort of bioreactor utilized in SSF, most commonly in research facility investigate for enzyme production, for lignin debasement for the application of organically pretreated fabric within the handle of biogas generation. It is additionally utilized in commercial processes in different businesses, such as the generation of matured nourishments such as tempeh and the generation of different chemicals. Usually due to its straightforward plan and ease of utilize *(Gordana Šelo and al., 2021).*

3.1.2. Factors influencing the FMS process

There are a few variables affecting the FMS prepare: Stickiness, inoculum, pH temperature and concentration of oxygen, the expansion of a source of carbon and/or nitrogen and the expansion of a particular actuating chemical for microorganism development and generation extracellular enzymes *(Gordana Šelo et al., 2021).*

3.1.3. Substrates used in FMS

The choice of substrate is more often than not decided by its fetched and accessibility, composition and its capacity to be changed over into a specific item through biochemical pathways. Agreeing to the objective (generation of the required protein, generation of phenolic compounds, acids natural or other important item craved, utilize for biofuel generation, utilize for nourish preparing), it is vital to know the composition of the substrate and select the suitable microorganism. In case the substrate does not contains not the specified sums of supplements, a few large scale and micronutrients are included for ideal development of microorganisms *(Farinas et al., 2015, Soccol et al., 2017).*

- Macronutrients (Carbon, Nitrogen, Oxygen, Hydrogen, Sulfur, Phosphorus, Mg 2+ and K+) are 10^{-4} M, whereas carbon within the center of development is the most source of vitality.
- Microelements (Zn^{+2}, Cu^{+2}, Mn^{+2}, Ca^{+2}, Na^{+2}) and vitamins, hormones development and digestion system of antecedents are required in concentrations underneath 10^{-4} M *(Behera et al., 2019).*

3.1.4. The advantages and disadvantages of FMS

The various advantages and disadvantages are summarized in the following table:

(José et al., 2021).

Table 07: The various advantages and disadvantages of FMS.

The advantages	The disadvantages
-The absence of open water reduces considerably the volume of installations fermentation.	-The microorganisms used are limited.
-Higher productivity, lower cost (thanks to the use of good agro-industrial residues market as substrates).	-It is practically difficult to ensure the direct control of culture parameters such pH, humidity and concentration of nutrients, quite random.

-Less use of chemicals, less waste generation and less energy consumption**.**	-Microorganisms being inseparable from substrate, the biomass estimate is delicate.

3.2. Submerged Fermentation (FML)

Liquid fermentation employments free streaming fluid substrates, such as molasses and broth. This aging procedure is best suited to microorganisms such as microscopic organisms, form and yeast that require a tall dampness substance. An extra advantage of this strategy is that the decontamination of the items is simpler *(Subramaniyam et al, 2012, Azzouz 2016, Liu 2016).* The submerged maturation is by and large performed at the locate of a free fluid in which dissolvable substrates are and is the foremost commonly utilized innovation for the colossal run of chemical generation *(Namnuch et Thammasittirong, 2020).*

Table 08: Comparison between FMS and FML characteristics as described by several authors (*De Castro et al 2015, Costa et al 2018, Leite, P et al 2020*).

	Solid stat fermentation	**Liquid stat fermentation**
Temperature	-Complicated control (exchange of warm restricted by bolster, discuss and the nonappearance of open water).	-Easy control.
	-Hazard of angle arrangement within the center.	-Homogeneous temperature.
PH	-Complicated control.	- Easy control.
	-Fractional direction conceivable (expansion corrosive or fundamental arrangements, utilize of buffer frameworks: arrangements, media in control buffer).	-Regulation by adding solutions acid or basic.

Aeration	-Absent discontinuous or continuous.	-Necessary (homogenization of the medium + aeration for crops aerobic).
	- Limited shear stress.	-Shear stress important (strain limitation cultivable).
	-May limit the heterogeneity of middle.	-Rheology related problem media (type of substrate + culture mold).
Antifoam	-Not necessary.	-Necessary.
Sterilization	-Plausibility of working in non-sterile or semi-sterile condition due to moo Aw (confinement of contaminants and their improvement) and once the implantation of the strain performed.	- Necessary.
	-Constrained hazard of defilement, basically for strains with moderate development and particularly by other mushrooms.	
	- Pretreatment by cooking or steam treatment to kill the most contaminants (vegetation native).	- High risk of contamination.
Type of culture	-Culture mainly in batch.	-Cultures in batch, in fed-batch or in continue.
Microorganism	-Agency friendly multicellular and/or to organisms suitable for low Aw and strong osmotic pressure (fungi filamentous, superior).	-Agency friendly unicellular (bacteria, yeasts).
	- Favourable to wild strains.	- Favourable to mutated strains or genetically modified.

	- Pure cultivation and the possibility of mixed cultures with the United Metabolisms.	- Pure crops, not favourable to mixed cultures.
	- Heterogeneous culture.	- Homogeneous culture.
	- Inoculation in conidia or mycelium.	-Inoculation in cells or crop volume.
Product	- High productivity (yield similar or higher and/or time of crop shorter than MLF).	-Product (very) diluted .
	- Increased performance: stability higher (T, even extreme pH), wider optimum (T, pH), resistance to inhibition, affinity for the substrate.	
	- Expression of novel molecules; (rich protein profile).	
	- Concentrated fermented product if direct use and therefore no concentration requirement (liquid).	
Repression catabolic or degradation by proteases	- Low or non-existent.	-High.
Culture medium	-Heterogeneous.	-Homogeneous (strain, nutrients,

	- Abundant raw material and bonmarché.	metabolites).
	- Abundant raw material and Bonmarché.	- Expensive raw material.
	- Insoluble solid polymers.	- Soluble or in the form of fines suspended particles.
	- Limited pretreatment or non-existent (agricultural waste).	-Pre-treatment sometimes necessary.
	- Amount of substrate high (high concentration, but problem of distribution of nutrients; risk of a high concentration of inhibitors).	- Limited substrate quantity (low concentration).
	- Limiting amount of water.	- Water quantity not limiting.
	- Not limiting air quantity.	- Limiting amount of air.

4. Optimization of protease production

Optimization alludes to the advancement of the biochemical prepare to get an item solid and environmentally cheaper. Plan reasonable generation media and conditions (temperature, pH and brooding time) is critical to progress efficiency and proficiency of bioactive microbial metabolites through maturation forms (*Naddem and Dimesh, 2016*). There are distinctive exploratory plans utilized for metabolite generation eminently proteins (*Hamma and Saoudi, 2016*).

4.1. Optimization through genetic engineering

The point of cloning the qualities of bacterial proteases was primarily to over production of protein for different commercial applications in segments nourishment, cleansers and

pharmaceuticals, hereditary adjustment coordinate (biotechnology) envelops the foremost exact

strategies to optimize microorganisms for the generation of proteins. These strategies are utilized to get tall surrender maker organizations.

The destructiveness of a few microscopic organisms is connected to the emission of a few proteases extracellular. Cloning the qualities of these organisms has been examined to get it the premise of their pathogenicity and to create comparing medicines play a critical part in cell physiology. Speaks to protease genes soluble clones completely different microorganisms.

Biotechnology too gives the apparatuses to exchange a grouping hereditary of a plant, creature or microorganism from which the generation on a commercial scale isn't satisfactory towards a microorganism that includes a secure history of protein generation for nourishment.

The maintained advance of present day hereditary building depends on a number of imperative specialized revelations: cloning, cloning qualities and sequencing DNA. (Lesser, David Nash,.2009).

4.2. Optimization by statistical plans

4.2.1. Experimental plans of Plackett and Burman

Burman Plackett could be a exceptionally compelling screening strategy to recognize noteworthy variables among numerous components that impact a handle utilizing a few tests *(Edulis et al., 2020).* This arrange permits to think about a few components at once all lessening the number of tests and communicating generation agreeing to a demonstrate This strategy is considered a dependable, reproducible and cost-effective device. ; it is utilized in optimizing the generation of diverse proteins such as α-amylase by Aspergillus oryzae and Rhizopus oryzae *(Francis et al., 2003; Ait Kaki El-Hadef El-Okki and al.,2017),* chitinase by Streptomyces griseorubens *(Gasmi and Kitouni, 2016)* and proteases by Aspergillus oryzae and Bacillus subtilis . These proteases are created on crops based on common substrates from rural squander and agri-food businesses *(Belmessikh and al., 2013; Sathishkumar and al., 2015).* These networks make it conceivable to assess the impact of k components at two levels on the considered reaction with a number of tests N, such as N = k+1, so with a least of tests. The arrange tests of Plackett and Burman comprises of square networks called Hadamard matrices containing as it were components break even with to +1 or -1, developed by circular change from a fundamental generator.

4.2.2. Box Plans – Behnken

It may be a arrange that can incorporate up to twelve tests, to which can be included focuses within the middle. The test focuses of the Box-Behnken arrange are appeared in middle edges of each side of a 3d shape.

These plans don't have straightforward arrange generators (they are built in combining two-level figure plans with fragmented piece plans), and have complex perplexed intuitive.

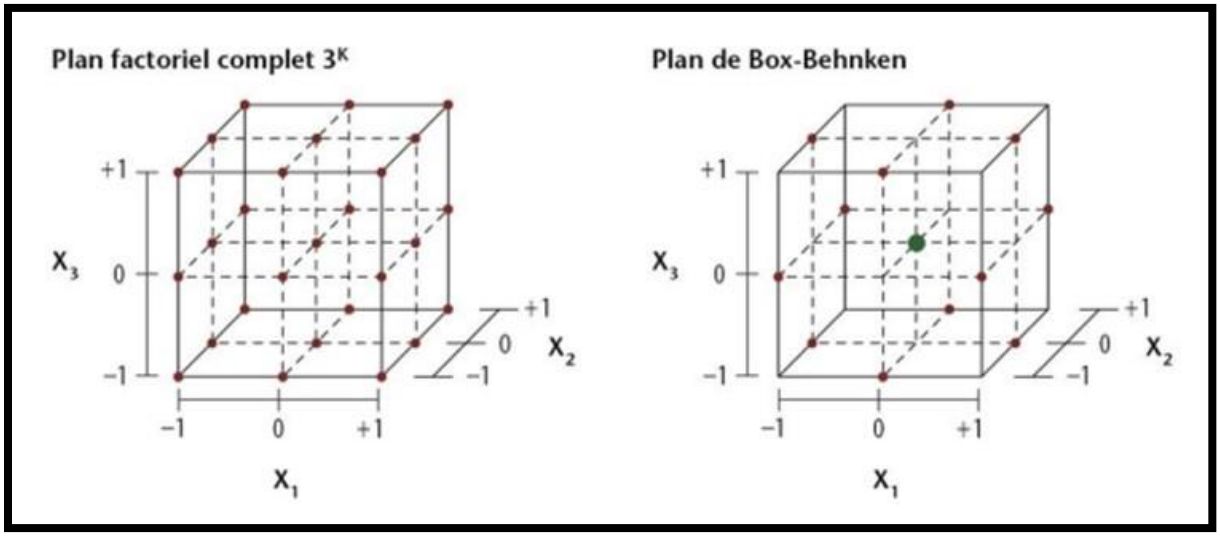


Figure24: Graphic illustration of a factorial plan composed of 3 factor Box-Behnken

(louis et al., 2018).

4.2.3. Plans of Box and Wilson

This test plan was presented by Box and Wilson (1951) the encounter is produced on a circle centered around a central point. The number levels of diverse components is minimized. The central point calculation is rehashed over and over in arrange to maximize the precision of expectation around the point assumed ideal.

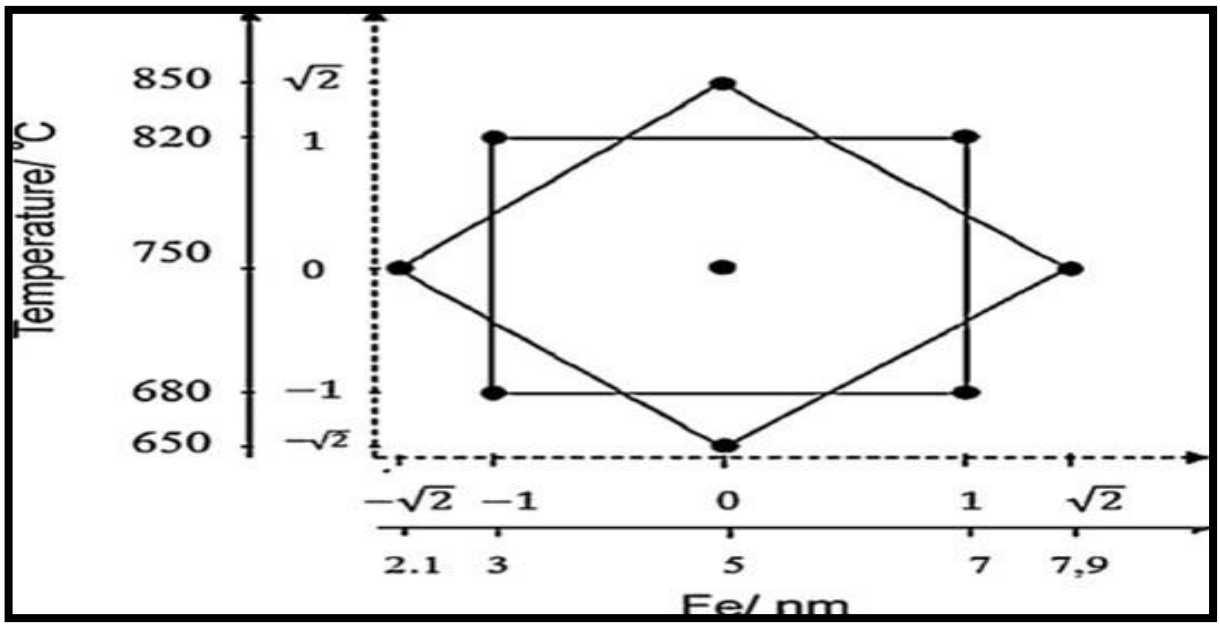


Figure25: Box-Wilson experimental design (Hussain, 2014).

4.2.4. OFAT method (one factor at a time)

The one-factor-at-a-time strategy (OFAT) is connected to select the energetic variable. This plan depends on the think about of a figure whereas putting the other steady factors *(Abou Taleb and al., 2018).* It could be an organized approach to consider the impacts of concurrent alteration of variables by constraining the number of tests *(Gallo, 2017).*

The calculate can be: a vital physical or dietary parameter for the control of micro-organism development and protein surrender *(Bhardwaj et al., 2019).*

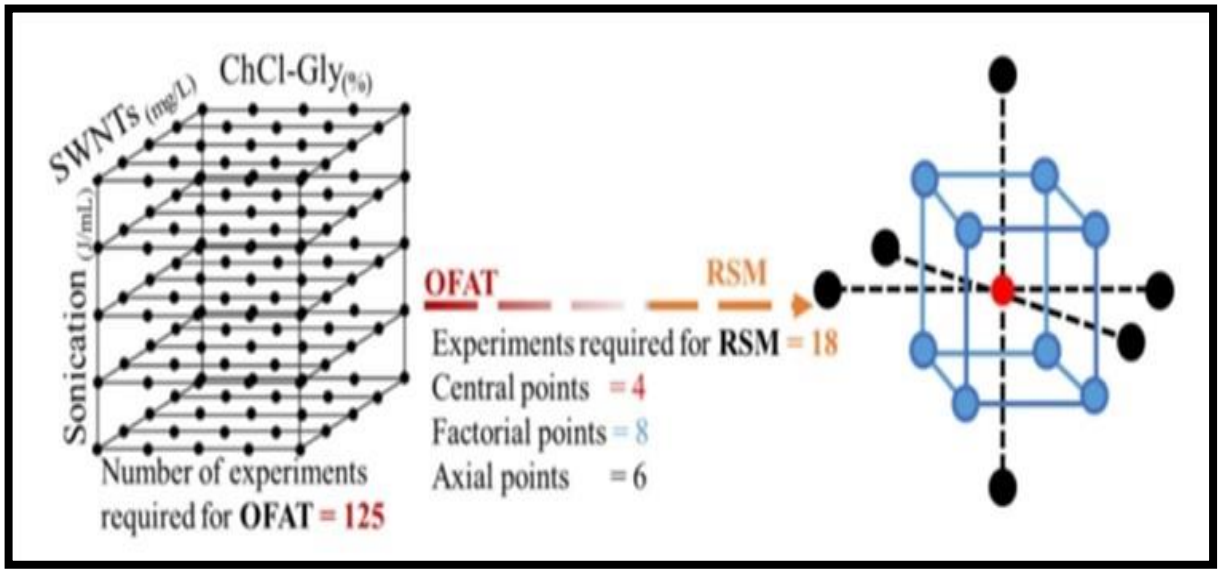


Figure26: One-factor-at-a-time-OFAT-for-three-variables *(Qammer Zaib,.2020).*

4.2.5. Composite Centered Plane (CCD)

Each microorganism has its claim conditions for greatest generation of wanted metabolite. In this manner, the optimization of the components of the medium ought to be performed to keep up a adjust between the different components, minimizing the sum of substances not utilized at the conclusion of aging *(Naddem and Dimesh, 2016).*

The centered composite plan is an compelling test instrument for the distinguishing proof of variables, the ponder of their intuitive and the choice of ideal conditions for a multivariable framework.

- A few reports on the centered composite plans are utilized for the optimization of the multi-enzyme generation Generation of polygalacturonase by Aurebasidium pullulans *(Bennamoun, 2017).*
- Protease generation by Aspergillus oryzae *(Benkahoul et al 2017).*
- Generation of amylopullulanase by Clavispora lusitaniae ABS7 *(Dakhmouche, 2016).*

The centered composite arrange was initially presented by Box and Wilson in 1951. It wasreceived to decide the optima of critical factors for greatest reaction. This arrange is shaped of three unmistakable parts to produce a break even with number of combination medications at 2k + 2.k + N.

- A two-level factorial plan (-1, +1), more often than not part from k calculate, which shapes a total 2k factorial arrange.
- 2.k focuses in stars, with levels (-α) and (+α), The esteem of the α may be a work of the number figure k.
- N redundancies of the center point (level 0). The esteem of N depends on the number of components k.

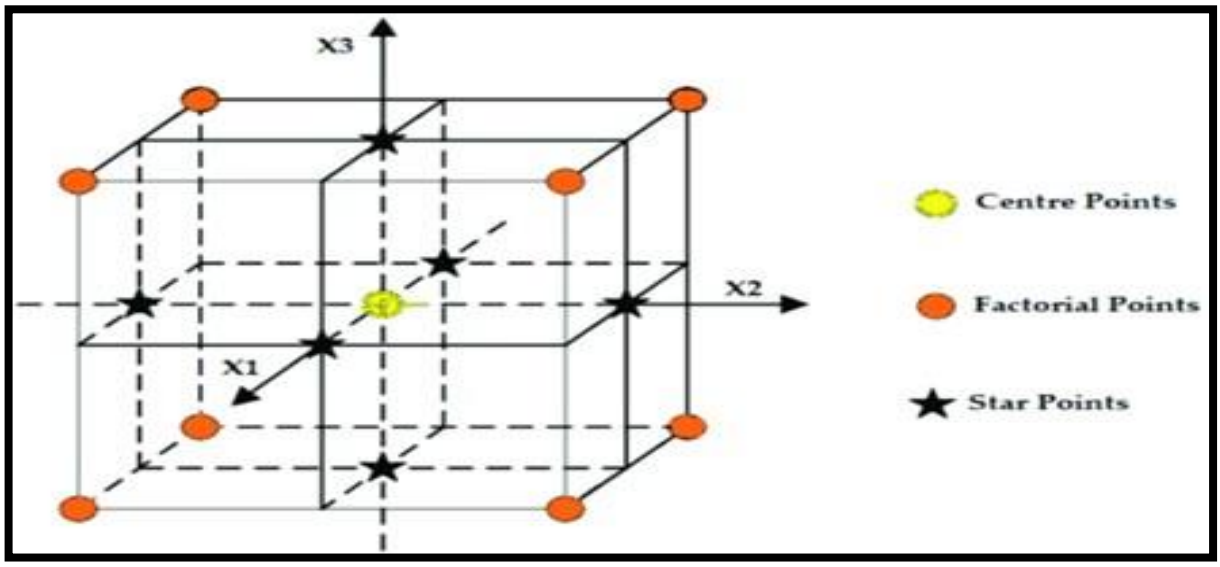
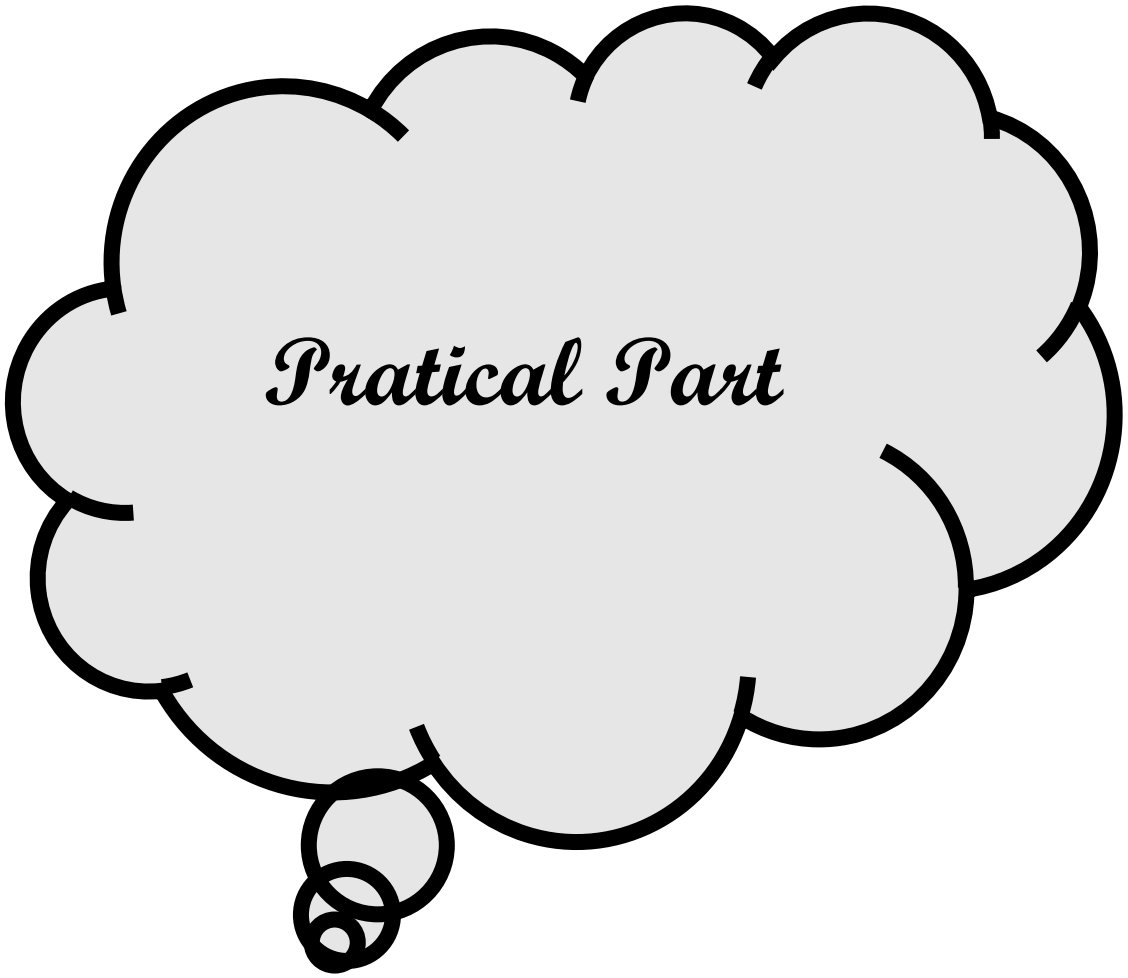


Figure27: The layout of centered design for central composite design CCD

(Tiwari et al., 2021).


Pratical Part

Chapter 04

Materials & Methods

This study is carried out at the level of the Engineering Laboratory Microbiology and Applications University of the Brothers Mentouri Constantine 1.

- This work is divided into three stages:
- ✓ **1st** step: Reactivation of the yeast selected for protease production.
- ✓ **2nd** step: Fermentation and optimization of growing conditions.
- ✓ **3rd** step: Statistical analysis of the results.

1. Microorganisms

1.1. Reactivation of *Rhodotorula Sp2* yeast

1.1.1. Exchange of yeast isolation

Yeast isolation is done from stock solution: 10 g yogurt and tween 80 to 0.45% added to 90 ml of sterile distilled water. Then shake well.

1.Yogurt.

2.Tween 80.

3.Distilled water.

1.1.2. Preparation of dilution

- After homogenization, prepare a series of decimal dilutions of stock solution up to10^{-7} *(Jerome et al., 2004).*
- Sink the medium YGC sterile in petri dishes.
- After solidification of the medium 100µl of each dilution are spread separately on the medium surface with rake *(Tortora et al., 2003).*
- Incubate at 25°C for 2-7 days.
- Daily observation of the macroscopic and microscopic aspect of isolates are realized.

1.1.3. Purification of strains

The purification of yeast as carried out by the striated method on YPGA (Appendix 1). Strain incubation is performed at 25°C for 2 to 3 days *(John and Michael, 2007).*

1.1.4. Preservation of strains

Pure strains are stored on YPGA medium (Appendix 1) at 4°C for a short time and on inclined medium of YPGA + Glycerol at -20°C for a long time.

1.1.5. Strain selection

- The first selection of yeast strains is based on rapid growth on YPGA at 25°C.

Figure 28: *Rhdotorula Sp2* growth on YPGA at 25°C.

1.2. Counting

Viable cell counting is often required during manipulations or of microbiology experiments to determine the number of cells present in a culture, both on liquid medium and also suspended in a liquid medium. This may include bacteria, yeasts, molds; unicellular algae, etc. In particular, such a counting is essential in cell metabolism experiments to determine the number of molecules consumed or produced (Glucose, Oxygen, Carbon, Dioxide carbon, etc.) by a single cell, for example by respiration, fermentation or photosynthesis *(Guezlane et al., 2010)*.

Cell counts are performed by direct counting using a Thoma cell (0.1 mm, 1/400 mm^2) (Figure …). In optical observation (200). A range of $2*10^6$ to $2*10^8$ spores and establish for a spore curvature. The optical density of spore suspension is determined at 600 nm.In our case we use 10^7spores and establish for a spore curvature.

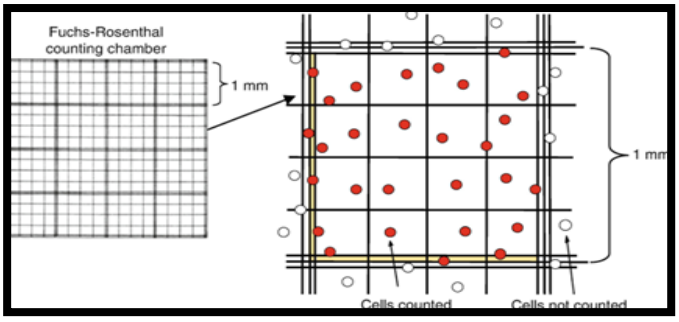


Figure 29: Thoma cell. **Figure 30**: Thoma cell counting chamber.

2. Protease production

2.1. Identification of enzymatic activities:

The medium used to demonstrate this activity are:

- 20 g/l agar and sterilized in an autoclave at 120°C for 20 min.

- Cool 40°C in a water bath.

- Add 100g/1L of skimmed milk (Juszczyk et al., 2005).

Figure 31: Preparation of the medium.

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

2.2. Wastes used

2.2.1. Tomato waste

Tomato clears out are by-products of tomato generation and a potential source of proteins. Portion of these proteins are proteases that carry out imperative capacities for the plants *(Yafei et al., 2024).* Tomato waste represents about 10-30% of the weight of fresh fruit *(King and Zeidler, 2004)*; they consist of 33% seeds, 27% skins and 40% pulp in addition to unprocessed green tomatoes, sometimes mixed with leaves. In Algeria, production annual tomato residues is estimated at 1,305,000 tones/year (FAO, 2009). Waste from dried tomatoes contain 44% seeds and the rest 56% skins and pulp.

To prepare tomato peel powder simply preheat the oven to 100°C and place the tomato skins on a baking sheet lined with baking paper, Bake for 2 hours. When the skins are dry, we keep them in an airtight jar (**Figure 32**). We can then chop them with a pepper mill or with a mortar pestle as and when used.

Figure 32: Steps of the preparation of tomato peel powder.

2.2.*2. Orange waste*

According to Robert et al., (1999) it is constituted from the outside to the inside of:

. Bark: consists of two parts:

- The epicarp: it is the colored part called «Flavedo». It represents 8 to 10% of the fruit, it contains essential oil glands, carotenoid pigments, vitamins etc.

- The mesocarp: the internal mesocarp is called «Albedo». It represents 12 to 30% of the fruit, from whitish color, it contains cellulose, soluble sugars, amino acids, vitamins and pectin.

. Pulp: it is the edible part representing 50 to 80% of the fruit, it is formed by the endocarp, consisting of juice-containing vesicles, are separated by quarters of which the number ranges from 9 to 11.

. Seeds: they represent 0-4% of the fruit and have a high oil content**.**

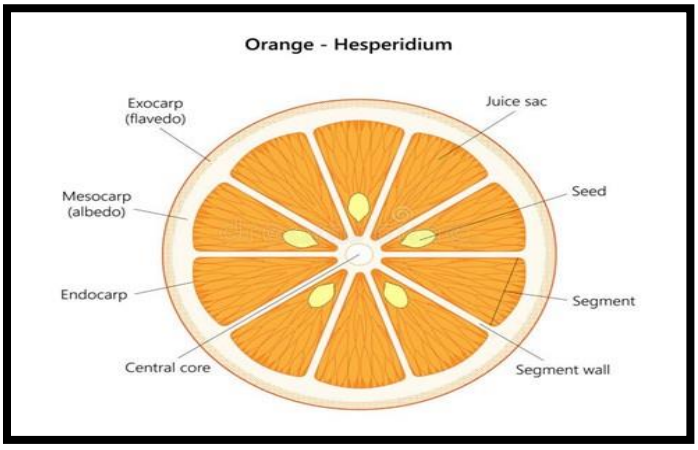


Figure33: Morphology of orange.

The orange waste supplied by the UNAJUS of SKIKDA is practically contiguous to the endocarp since it is removed from the chain to extract essential oils (Figure…).

2.2.3. Date waste

Date palm (Phoenix dactylifera L.) may be a socio-economically andcustomarily vital edit within the Center East and North Africa (MENA). Dates are wealthy in carbohydrates and other supplements such asminerals, proteins, fats, and vitamins. As a result, dates have long beenan vital portion of most Center Eastern diets *(Siddiq et al., 2013).* In 2017, the FAO detailed that 8.17 million tons of date natural product werecreated around the world *(Oladzad et al., 2021).*

We use Daglet-Nour date (**Figure 34**) varieties , from the plam of region, Algeria.

Figure 34: Daglet-Nour date, Algeria.

2.2.4. Wheat Bran

Wheat consumption is expected to increase globally by 2028 to reach around 330 million tones and will continue to be used mainly for food *(OECD-FAO, 2019).* According to Onipe et al., (2015) grain is composed of 13-17% of bran, 2-3% germ and 80-85% starch-rich endosperm, Outer layers are all parts of the sound. In the food industry, wheat bran is used as an alternative to substrates used in the fermentation process *(Pandey, 1992)* as well as in the production of secondary enzymes and metabolites *(Prückler et al., 2015).* However, the main application of wheat bran concerns animal nutrition: because of its nutritional properties, it improves the nutritional quality of food products from animal sources *(Coda et al., 2014).*

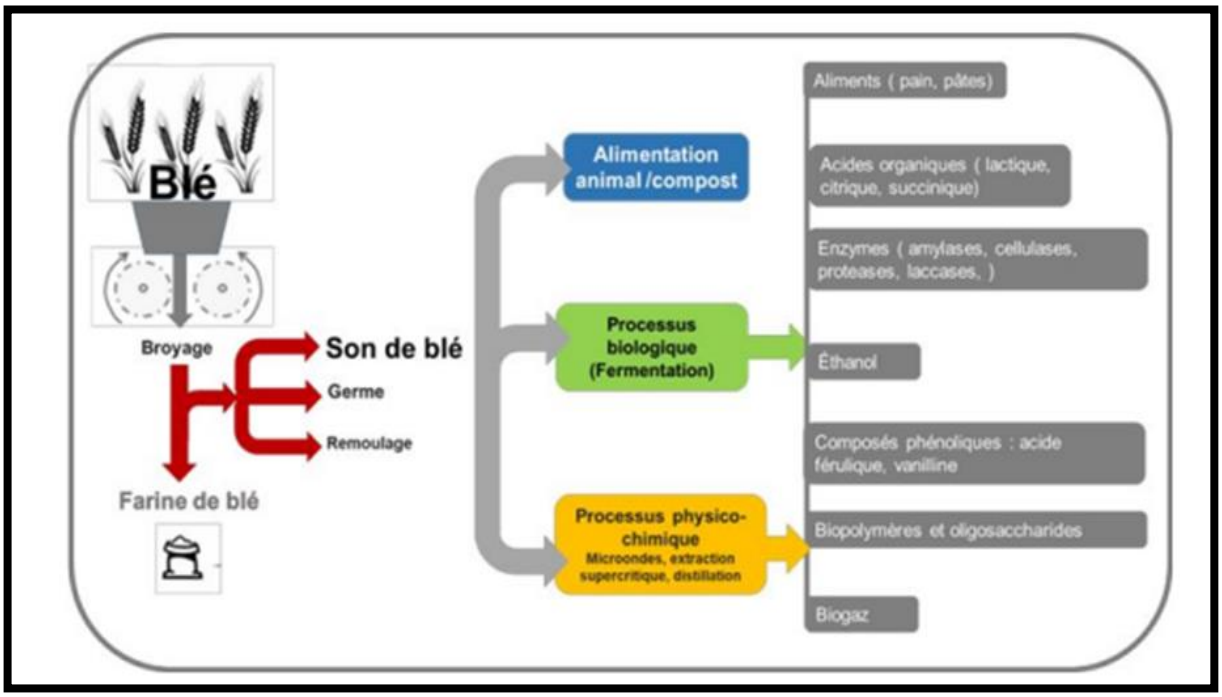


Figure 35: The different products of wheat bran (Author, 2020).

2.2.5. Lactoserum

Formerly called whey, whey is a by-product of the cheese industry and the preparation of caseinates *(Jouan, 2002).* Whey is a product discovered more than 3000 years before Christ, by Bedouins during milk transport. Acidification and the coagulation by heat caused the formation of a liquid phase above a milk curd *(DeWitt, 2001).* Whey represents 90% of the original volume of milk used in cheese making and is the main by-product . The term whey refers to the translucent, greenish-yellow liquid that separates from the curd *(Heslot, 1996)* after separation of caseins by acid coagulation or process enzymatic with rennet or chymosin *(Jouan 2002).*

Depending on the production process, different types of whey can be obtained. These lactoserums can be classified into two main categories according to the acidity of the liquid obtained *(Alais, 1984):*

- Mild whey: with acidity ranging from 15 to 22°D (pH=6.5). They come from the production of pressed and/or cooked or soft pasta *(Sottiez, 1990).*
- Acid whey: obtained in the manufacture of fresh pasta or in the casein production reaches 120°D, or a pH close to 4.5 *(Sottiez, 1990).*

To prepare our lactoserum :

- Put curd is drain to get lactoserum.
- Recover the lactoserum in a .... 100 ml.

- sterilized in an autoclave at 120°C for 20 min.

Corn-steep liquor

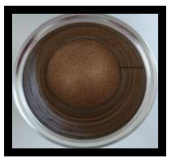
The corn-steep liquor is provided by SAIDAL (Médéa, Algeria) in liquid form. A by-product of starch extraction, it is widely used for its nutrient richness of microorganisms in fermentations in the production of penicillin for example. Before its use the corn-steep liquor is clarified by centrifugation to 15000 g for 20 minutes and the supernatant obtained is used in enrichment culture medium.

2.3. Chemical composition of wastes

Table 09: Chemical composition of wastes

(Tomato waste, Orange waste, Date waste, Wheat bean,and lactoserum).

Waste	Composition	Percentag	Referances
Tomato waste 	**Proteins** **Dry matter** **Total sugars** **Fatty matter**	**24,72 %** **93,03** **4,25** **26,20**	**Amalou et al., 2013**
Orange waste 	**Proteins** **Fatty matter** **Carbohydrates** **Organic acids** **Spring salts**	**6,99 %** **1,39 %** **57,69 %** **7,90 %** **3,35 %**	**Dakhmouche, 2001.**
Date waste 	**Humidity** **Dry matter** **Proteins** **Total sugars**	**10 %** **4,5 %** **3,22 %** **90 %**	**Bennamoun, 2017.**

Wheat bran 	**Humidity** **Dry matter** **Proteins** **Fatty matter** **Total sugars**	**10,95** **3,10** **4,12** **16** **2,3**	**Bennamoun, 2017.**
Lactoserum 	**Dry matter** **Matter mineral** **Total sugars** **Protein**	**6,5 %** **10,46 %** **71,53 %** **8,61 %**	**Benkahoul et al., 2017.**

2.4. Culture medium and fermentation conditions

The fermentation is carried out on liquid medium based on five types of wastes: Tomato waste, Orange waste, Date waste, Wheat bran and Lactoserum.

- ✓ The basic culture medium is prepared from 5 g dry of substart that is dissolved in 50 ml of distilled water with stirring until a homogenic solution is obtained.
- ✓ Before sterilization at 115°C for 20 min the pH of each Erlenmeyer is adjusted statistically.
- ✓ It is seeded with 10^7 spores/ml, then homogenized to avoid aggregation and incubated at 30°C for 2 days under a stirring of 150 rpm by the shaker.

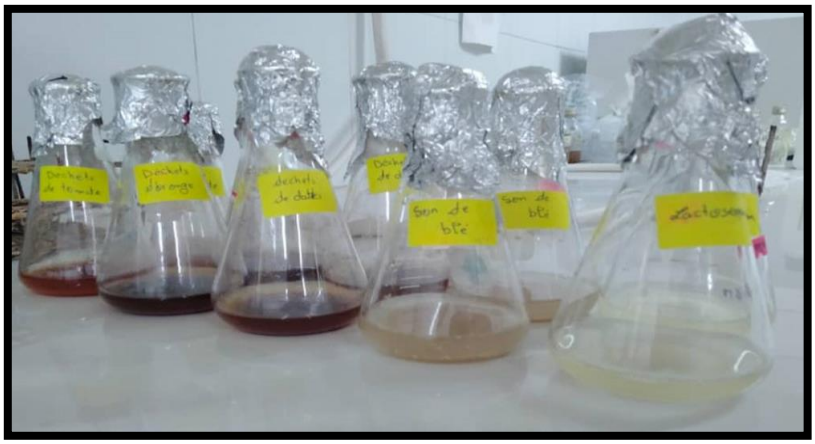


Figure 36: The 10 Preparations of liquid culture medium based on the 5 types of waste.

Finally, after fermentation the exo-cellular medium is separated from the biomass by a simple filtration with the utilization of a Whatman filter paper N°01 (**Figure 37**), The exo-cellular medium represents the raw enzymatic extract. The biomass is weighed in each Erlen and the exocellular medium undergoes an assay of proteolytic activity and proteins. The results obtained are then analyzed by the software.

Figure 37: Filtration process with the utilization of a Whatman filter paper N°01.

2.5. Culture medium based on orange waste and fermentation conditions

- ✓ The basic culture medium is prepared from 5 g dry orange waste that is dissolved in 50 ml of distilled water with stirring until a homogenic solution is obtained.
- ✓ The medium is enriched (according to the experimental design) and then distributed in 250 ml Erlen at a rate of 50 ml per Erlenmeyer.
- ✓ Before sterilization at 115°C for 20 min the pH of each Erlenmeyer is adjusted statistically.
- ✓ It is seeded with 10^7 spores/ml, then homogenized to avoid aggregation and incubated at 30°C for 2 days under a stirring of 150 rpm by the shaker.

Figure 38: The 12 Preparations of liquid culture medium based on orange waste.

Finally, after fermentation the exo-cellular medium is separated from the biomass by a simple filtration with the utilization of a Whatman filter paper N°01, The exo-cellular medium represents the raw enzymatic extract (**Figure 39**). The biomass is weighed in each Erlen and the exocellular medium undergoes an assay of proteolytic activity and proteins. The results obtained are then analyzed by the software.

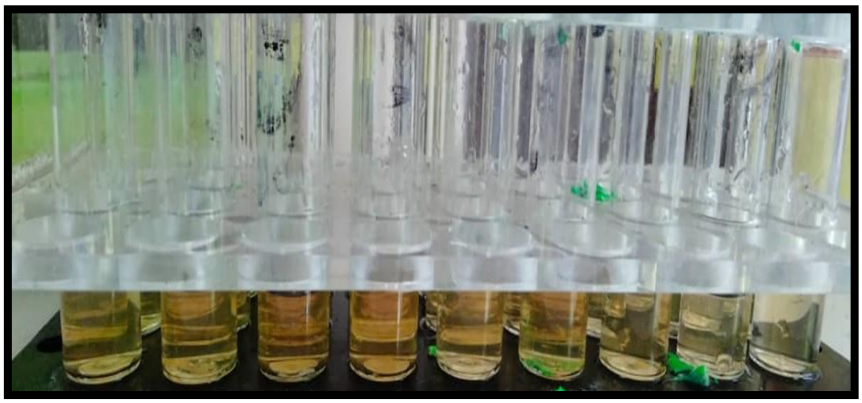


Figure 39: The different filtrates of liquid culture medium based on orange waste.

3. Protease assay

Protease assays are performed after each step, there are two types of assays: proteolytic activity assay and total protein assay.

Proteolytic activity is measured using the method described by AUBERGER et al (1995) and modified by MECHAKRA et al. (1999). The principle of reaction is as follows:

Proteases catalyze the hydrolysis of proteins and polypeptides to transform them into shorter protein fragments (polypeptides, simple peptides and free amino acids). TCA allows the precipitation of insoluble fractions. Filtration allows the recovery of soluble fractions, including tyrosine groups. The presence of tyrosine in the filtrate is translated into proteolytic activity by

a colorimetric assay according to the Anson method, (1938), using the Folin-Ciocalteu reagent. It reacts with these groups and with tryptophan to give by reduction a blue complex. The activity is calculated by reference to a calibration curve established using tyrosine as standard with concentrations between 0 and 100 g/ml. One unit (U) of protease is the equivalent of 1 g of tyrosine released for 1 h of time per ml of an enzyme solution with either casein or hemoglobin as substrate. Each dosage is done in duplicate.

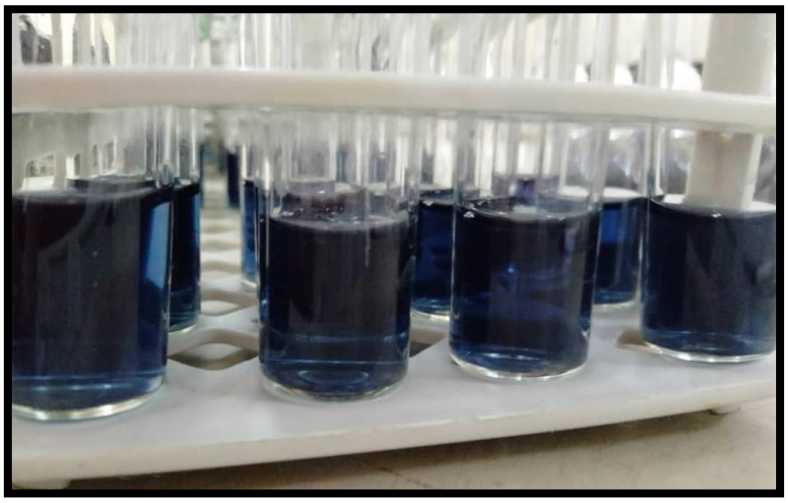


Figure 40: The result of AUBERGER's coloring method.

3.1. Solutions used

- Casein 2,5 % in Sodium Citrate (0,02 M).

- Stock solution 100 µg/ml Tyrosine.

- TCA 4%.

- Na_2CO_3 2 % in NaOH (0.1 N).

- Folin-Ciocalteux 1/3rd.

3.2. Proteolytic activity is measured in two stages

- The first stage is the enzymatic reaction, the reaction mixture of which consists of:

- 1ml of the raw extract.
- 1.5ml buffer of each pH (04 ;07 ;09).
- 2.5ml of the 2.5% casein solution dissolved in citrate sodium0.02M.

After incubation in a water bath at 40°C, the reaction is stopped by adding 2.5 ml of TCA (4%).

- The second stage: Colorimetric determination of enzymatic activity is carried out by mixing:

- 0,5 ml of filtrat.
- 2.5 ml Na2CO3.
- 0.25 ml of folin ciocalteu.

The mixture is stirred and left to stabilize at room temperature for 30 min.

After incubation at room temperature for 30 min absorbance reading a place to750 nm to the spectrophotomet**.**

3.3. Tyrosine calibration curve

Table 10: Tyrosine Calibration Curve Protocol.

Tubs N°	Temoin	01	02	03	04	05
Tyrosine concentration (µg/ml)	00	20	40	60	80	100
Volume of Tyrosine removed (ml)	00	0,1	0,2	0,3	0,4	0,5
TCA (ml)	0,5	0,4	0,3	0,2	0,1	00
2% Na2CO3 in 0.1N NaOH (ml)	2,5					
Shake well then let the mixture stabilize for 10 min.						
Folin reagent diluted (1/3th) (ml)	0,25					
After each addition of the Folin reagent and leave in the shade for 30 min at a minimum.						
Absorbance reading at 750 nm.						

- Proteolytic activity (µg/h/ml) = concentration deducted from calibration curve x dilution
  x 2 (to reduce sample volume to 1ml) x 2 (to reduce time to 1h).

- Activity in µ/ml =(Activity (µ/ml) x Volume used for extraction) / Dry mass.

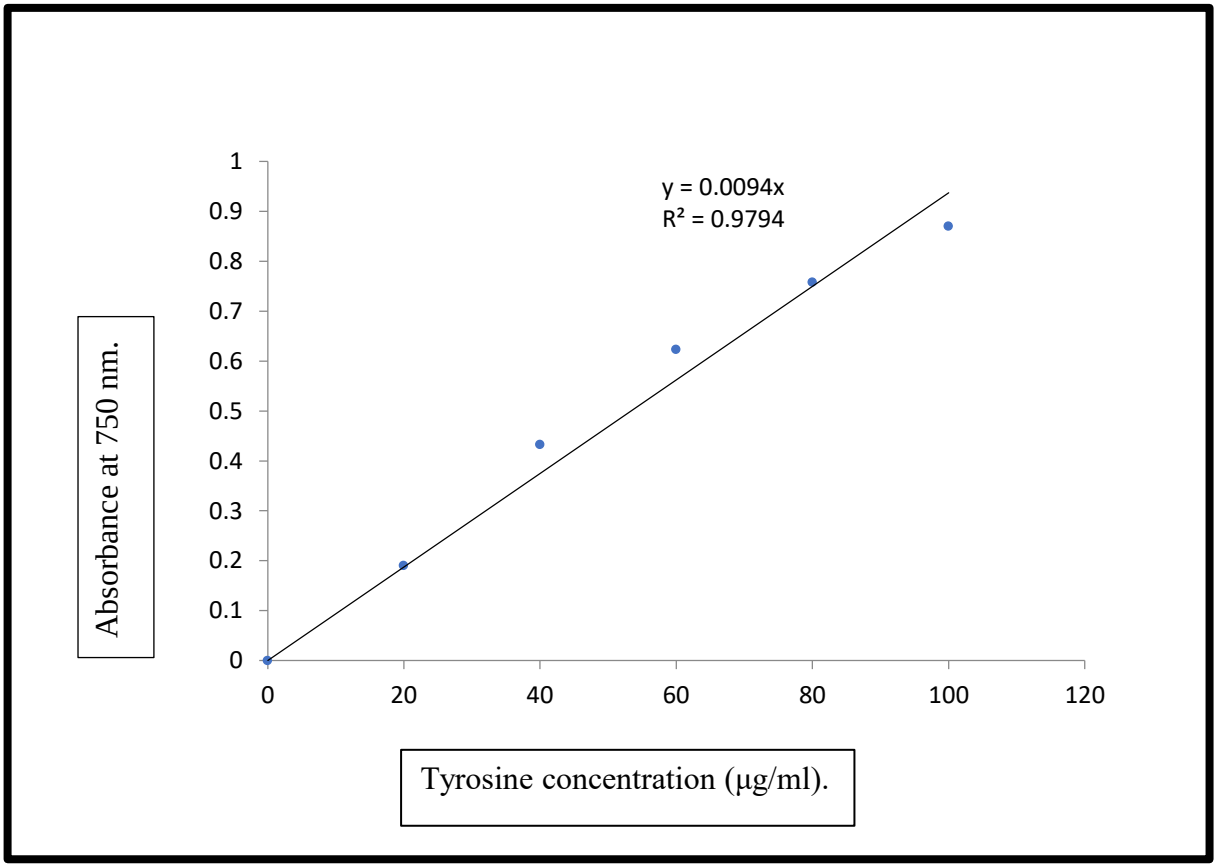


Figure 41: Tyrosine Calibration Curve Protocol.

4. Optimization Methods

Optimization refers to the advancement of the biochemical handle to get an item solid and biologically cheaper. Plan reasonable generation media and conditions (temperature, pH and incubation time) is vital to progress efficiencyand proficiency of bioactive microbial metabolites by means of fermentation forms *(Naddem and Dimesh, 2016)*. There are diverse experimental designs utilized for metabolite generation eminently proteins *(Hamma and Saoudi, 2016).*

4.1. OFAT (one factor at a time) method

The one-factor-at-a-time strategy (OFAT) is connected to select the energetic variable. This plan depends on the think about of a figure whereas putting the other consistent factors (*Abou*

Taleb et al., 2018). It may be an organized approach to ponder the impacts of synchronous alteration of variables by restricting the number of tests (*Gallo,2017*).

The figure can be a vital physical or dietary parameter for the direction of micro-organism development and chemical surrender (*Bhardwaj et al., 2019*).

4.2. Plackett and Burman Design

Burman Plackett may be an exceptionally viable screening strategy to distinguish noteworthy variables among numerous variables that impact a prepare utilizing a few *tests (Edulis et al., 2020).* This arrange permits to consider a few variables at once all lessening the number of tests and communicating generation agreeing to a demonstrate this strategy is considered a dependable, reproducible and cost-effective device; it is utilized in optimizing the generation of diverse chemicals such as α-amylase by *Aspergillus oryzae* and *Rhizopus oryzae (Francis et al., 2003; Ait Kaki El-Hadef El-Okki andal.,2017),* chitinase by *Streptomyces griseorubens (Gasmi and Kitouni, 2016)* and proteases by *Aspergillus oryzae* and *Bacillus subtilis* . These proteases are delivered on crops based on normal substrates from rural squander and agri-food businesses (*Belmessikh et al., 2013; Sathishkumar et al., 2015*).

These matrices make it conceivable to assess the impact of k components at two levels on the examined reaction with a number of tests N, such as N = k+1, so with a least of tests. The arrange tests of Plackett and Burman comprises of square matrices called Hadamard matrices containing as it were components break even with to +1 or -1, built by circular change from an essential generator (**Figure 42**).

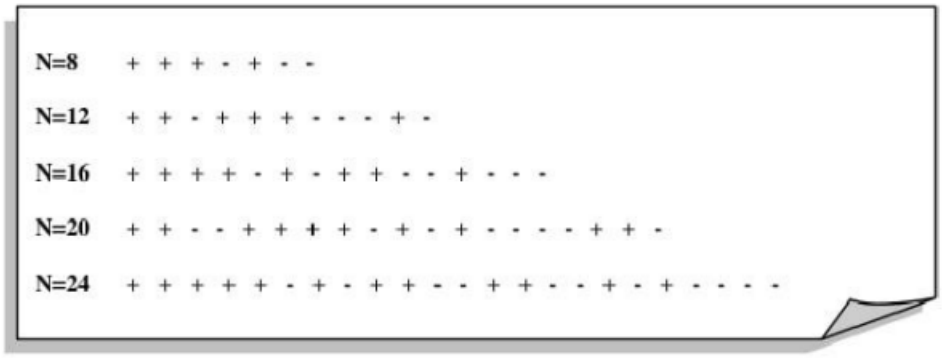


Figure 42: Matrix generator for N=8, N=12, N=16, N=20, N=24 tests

(Plackett and Burman (1946)).

In our case, the experimental plan adopted is that of Plackett and Burman (1946) to select influencing factors the production of proteases. A matrix of 11 factors (7 real factors and 4 unreal factors or errors) and 12 experiments were used. It's a square matrix, so containing only elements equal to +1 or –1, the last combination being always taken at the lower level -1 (**Table 10**). This matrix is obtained by circular from a basic generato

Table 11: Plackett and Burman experiment matrix for 11 factors with 12 Combinations.

	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

- **(+1) :** Higher level of factor.
- **(- 1) :** Lower level of factor.

The number of real factors is hence diminished to N-4, the think about of 7 variables which are: Glucose (A), Sucrose (B), Yeasy extrat (D), Corn Steep Liquor (E), Tween 80 (G), Na NO_3 (H), Mg SO_4 (J) (**Table 11**).

Table 12: The different factors studied and their corresponding concentrations.

Factors	Factors	Higher level (+1)	Lower Level (-1)	Unite
Glucose	A	1%	0%	%
Sucrose	B	1%	0%	%
Error	C	----	----	-
Yeast Ex	D	2%	0%	%
Corn steep Liquor	E	2%	0%	%
Error	F	----	----	-
Tween 80	G	1%	0%	%
Na NO_3	H	0,3%	0%	g/l
Error	I	----	----	-
Mg SO_4	J	0,1%	0%	g/l
Error	K	----	----	-

The effect of each variable is determined as a difference between the average of responses at the higher level (+) and the average of the response values at the level lower.

Chapter 05

Results & Discussion

This part presents all the results obtained on the study of the optimization of the proteases by *Rhodotorula* yeast. For this we tested the following factors the different substrates (Tomato waste, Orange waste, Date waste, Wheat bran, Lactoserum) in the liquid medium. For the purpose of selected what type of protease has occurred a dosage of proteolytic activity is determined at different pH (4,7 and 9) is also achieved.

Subsequently a statistical approach based on the plans of Plackett and Burman (1946) in order to select factors influencing the production of acid protease.

I. Qualitatif study of proteolytic activity

The results obtained after incubation on milk gel for 3 days, allowed the appearance of the transparent halo (clear ring) resulting from the degradation of milk agared by the exoproteases that occurs around the *Rhodotorula sp* colonies. This reaction hydrolysis yields usable soluble amino acids *(Clarke and Steel, 1966).* Diameter of this zone (**Figure 43**) is closely related to the amount of extracellular proteases produced by *Rhodotorula sp* enters. The lysis zone was 1.5 to 2 mm in diameter.

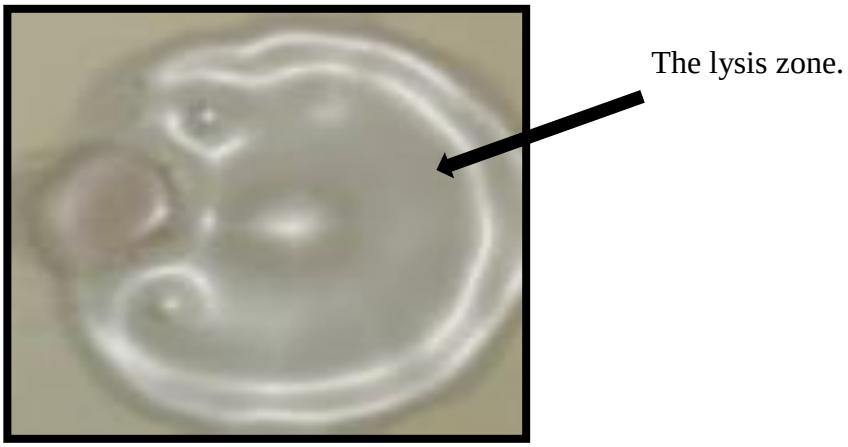


Figure 43: Demonstration of proteolytic activity of *Rhodotorula sp* on milk medium gelose (03 days of culture).

II. Quantitatif study of proteolytic activity

II.1. Wastes Composition

The composition of the wastes used in the preparation of crop media is presented in **Table 09**. The chemical compositions of the wastes used are summarized in **Table 09**.

Table 09 shows the dry matter content of tomato waste (93.03%). It also shows a rate of 26.60% fat, a rate of 24.72% protein a total protein is very low.

Table 09 shows the richness of orange waste in carbohydrates (57.69%) as reported by Souci et al. in 1994 (44%). It also shows a mineral content of 3.35%, comparable to that of Nouel and Combellas (1999) which report a content of 3.9%. The total protein content is very low compared to that of the bibliography. Indeed, the value determined on our samples represents 6.90%, almost half of the result obtained by Nishio and Nagai (1981) for the composition of the whole orange. These results show the richness of these wastes in carbohydrates and minerals and encourage their use as a base medium in yeast culture and therefore the production of enzymes.

For dates, 90% soluble sugars, 4.5% dry matter, and 3.22% total protein were found (**Table 09**). As in our study, the bibliography indicates the richness of dates in sugars and their low content of protein compounds (Vayalil, 2012; Mkaouar and Kachaou, 2013). They are particularly used as a fermentation substrate for their rich carbon source *(Ait Kaki et al., 2017).*

For whey, the composition indicates a rate of 71.53% for soluble sugars, 10.46% for mineral matter and 8.61% for proteins. This by-product of the dairy industry is characterized by its richness in lactose and mineral salts *(Alais, 1981; Sienkiewicz and Riedel, 1990).*

Table 09 also mentions the composition of Lactoserum where total soluble sugars represent 8.31%, dry matter 45% and protein 50.44%. The literature reports varying results depending on the source of corn steep liquor *(Hull et al., 1996; Gao and Yuan, 2011)* but all references present this industrial by-product as a good source of nitrogen encompassing a wide variety of amino acids *(Xiao, et al., 2013).*

II.2. Study of the production of proteolytic enzymes on FML

The production of proteolytic enzymes by FML is tested on different substrates: tomato waste (M1), orange waste (M2), date waste (M3), Wheat bran (M4) and lactoserum (M5). The determination of proteolytic activities is carried out at different pH: acid (pH 4), neutral (pH 7) and alkaline (pH 9).

The results are presented in **Figure 44:**

- There is an estimated maximum production of acid protease at **17555,55 U/h/ml** obtained on medium M2 (Orange waste). The lowest amount of enzyme is **10711,11 U/ml** is obtained with medium M5 (Lactoserum).
- There is an estimated maximum production of neutral protease at **17422,22 U/ml** obtained on medium M3 (Date waste). The lowest amount of enzyme is **7644,44 U/ml** is obtained with medium M5 (Lactoserum).
- There is an estimated maximum production of alkaline protease at **18400U/h/ml** obtained on medium M2 (Orange waste). The lowest amount of enzyme is **6888,88U/ml** is obtained with medium M4 (wheat bran).

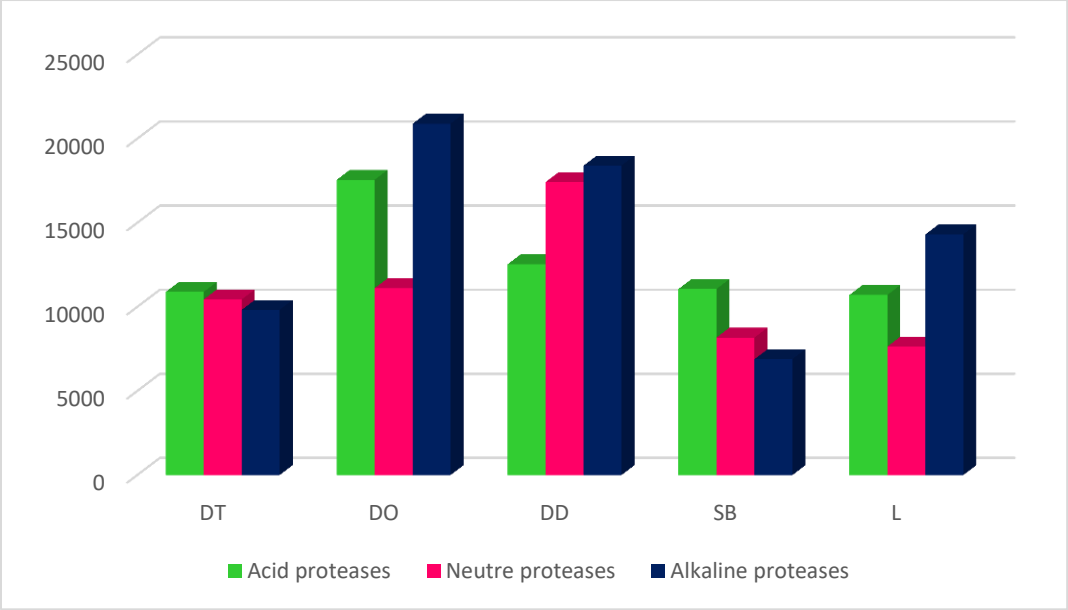


Figure 44: Effect of different substrates on protease production (acid, neutral and alkalin) by FML by Rhodotorula Sp.

III. Optimization of culture conditions for acid proteases production by experimental planning

III.1 Selection of growth and production factors by the statistical design of Plackett and Burman (1946)

The results of experiments carried out according to the plan of Plackett and Burman give the results of the biomass and the proteolytic activity represented in the **Table 12** The best growth is obtained in Test N °5 containing corn-steep liquor and salts: Na NO3 and Mg SO4. While the best proteolytic activity is observed in test N °3 containing glucose, yeast extract, Tween 80 and salts: Na NO3. However, the lowest activity is obtained in the base medium (test N °8).

Table 13: Effect of tested factors on neutral protease production (Dsign of Plackett-Burman).

	Factors											Biomass	Proteolitic Activity
	A	B	C	D	E	F	G	H	I	J	K		
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	1,205	58622,22
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	1,508	52088,88
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	1,611	**66355,55**
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	1,481	60888,88
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	- 1	**1,819**	51377,77
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	1,538	48844,44
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	1,021	41022,22
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	0,838	38533,33
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	1,206	49688,88
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	1,141	45866,66
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	1,417	47333,33
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1,285	44977,77

The effect of each factor is tested by calculating the correlation coefficient and the level of statistical significance on each response is determined by the probability test (P):

- P<0.05: the result is significant, so the factor is retained.
- P>0.05: the result is not significant, so the factor does not have a significant effect on answer which excludes it from the study.

The statistical analysis (**Table 13**) of the production of the acid protease is explained significant by Sucrose (p=0.008) and Yeast extrate (p=0.007). Non-significant factors reported by Glucose (p=0,233), Corn-steep Liquor (p=0,440), Tween80 (p=0,289), Na NO_3 (p=0,223) and Mg SO_4 (0,265).

Table 14: Statistical study of the effect of the tested factors on the production of acid protease.

Terme	Coeff	Coef Ert	T value	P Value	FIV
Constante	10278	203	50,57	0,000	0,00
C1	-285	203	-1,40	0,233	1,00
C2	-1004	203	-4,94	0,008	1,00
C4	1019	203	5,01	0,007	1,00
C5	174	203	0,86	0,440	1,00
C7	248	203	1,22	0,289	1,00
C8	293	203	1,44	0,223	1,00
C10	-263	203	-1,29	0,265	1,00

- **Expression of results**

The results of proteolytic activities can be presented by a polynomial equation of first degree:

$Y = \beta 0 + \beta 1C1 + \beta 2C2 + \beta 3C3 + \beta 4C4 + \beta 5C5 + \beta 6C6 + \beta 7C7.$

- **Proteolytic activity equation**

Proteolytic activity = 10278- 285**C1**- 1004**C2**+ 1019**C4**+ 174**C5**+ 248**C7**+ 293**C8**- 263**C10.**

- **Equation reduced**

Y = 10278- 285**Saccharose** +1019**Yeast extrat.**

The statistical analysis indicates a value of the coefficient of determination of R^2= 93.43 %for this model. This means that 93.43% of acid proteases production is explained by this model and that 6,57 % therefore remain unexplained.

- **Conclusion**

Statistical analysis of the results of this design and modelling using linear regression multiple allowed to select a significant positive or negative effect on the activity proteolytic namely Glucose , Sucrose, yeast extrate, Corn steep Liquor, Tween80, Na NO_3, Mg SO_4.

The production of acid protease is clearly influenced by sucrose and yeast extrate. The optima of these selected factors will be determined previously by the use of another design: composite centered plan of Box and Wilson (1951).

The P values used as a tool to check the meaning of each of the coefficients which in turn are needed to understand the interaction model the P-values below 0.05 indicate that the P-values of coefficients are significant. The value of the t-test is large and the more the value of P tends to 0 indicates the meaning of the corresponding coefficient and variables with coefficients negligible are omitted.

Table 14 shows the significant coefficients of the second-degree polynomial of activity proteolytic are determined by the student test (t) and (p) value.

Table 15: Regression results of the second-degree polynomial model for optimizing the production of acid proteases.

Source

Model	7	28473425	4067632	8,21	0,030
Linear	7	28473425	4067632	8,21	0,030
C1	1	976011	976011	1,97	0,233
C2	1	12089245	12089245	24,39	0,008
C4	1	12448392	12448392	25,12	0,007
C5	1	363590	363590	0,73	0,440
C7	1	738971	738971	1,49	0,289
C8	1	1027365	1027365	2,07	0,223
C10	1	829849	829849	1,67	0,265
Error	4	1982622	495655	-----	-----
Total	11	0456046	-----	-----	-----

The relevance of the model is assessed through several technical factors such as the coefficient of determination R^2, fitted determination coefficient (Adj), coefficient of predicted determination (Predicate), Fisher test F and P-values for linear, quadratic and intra effects of the factors used (**Table 15**).

ANOVA is an important statistical tool, widely used to further verify the model convenience.The coefficient of determination is used as an index to confirm the accuracy and quality of the fit of the model, it is generally between 0 and 1. When the closely approximates 1, the model strongly predicts the response. Several authors report whereas the coefficient should reach 0.80 for a good fit of the model (*Dragoï and Vasseghian, 2020*).

Statistical analysis indicates the value of the coefficient of determination = 0.9227 which implies that 92.27% of the variations in the neutral protease activity, the predicted value of 0.4806 (48.06%) indicates good agreement between experimental and predicted values.

The value of the adjusted coefficient of determination Adj = 82.32% represents a correction of the value of which allows the sample size and the number of variables used in the model (*Bennamoun, 2017*). The coefficient of determination Relatively high adjusted (Adj= 0.82) in this study explains good quality model description.

Table 16: Model overview.

S	R^2	R^2 (Ajust)	R^2(Prev)
704,028	93,49%	82,10%	41,41%

III.2. Pareto Charts

The Pareto diagram (**Figure 45**) graphically represents the design analysis results for acid protease production and illustrates the order of significance of the variables. It appears clearly that the parameters, sucrose moisture and yeast extract are significant for the production of acid proteases, as well as their mutual interactions C2 C4 the quadratic term C32 is indicated from 2.776.

According to the Pareto chart obtained, we note that the quadratic terms C1, C5, C7, C8 and C10 are not significant on the production of acid proteases.

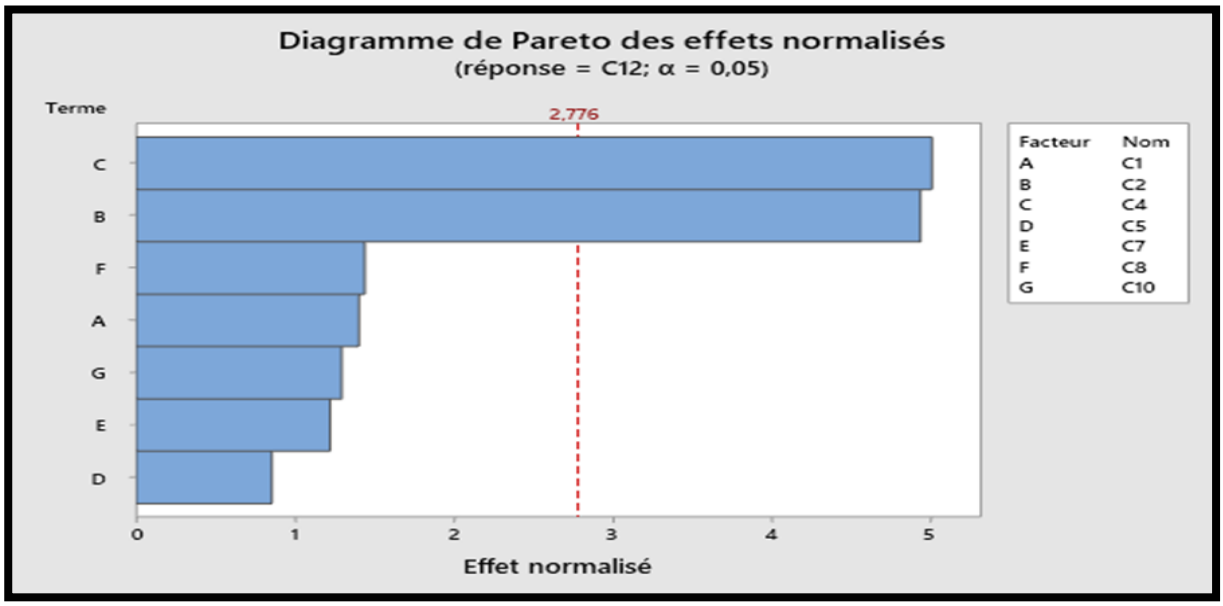


Figure 45: Pareto charter of standardized effects of operating parameters on activity proteolytic.

IV. Discussion

La production proteolytique est meilleure à pH 4. Ceci peut être expliqué par le fait que le pH acide convient mieux à l'activité de la protéase produite et nous pouvons déduire que la levure (*Rhodotorula sp2*) est productrice de protéase acides.

Orange waste medium enriched with yeast extract, starch and peptone is more efficient for protease production. This can be explained by the fact that the nitrogen source provided by orange waste and carbohydrates are insufficient for the growth of *Rhodotorula sp* and therefore for protease production. This enriched medium allows *Rhodotorula sp* to use Glucose (1%), sucrose (1%) as a single source of Carbon, Yeast extract (2%) as a source of nutrients and Nitrogen, Corn-steep Liquor (2%) as a source of Nitrogen, Tween 80 (1%) as a non-ionic surfactant, Na_2NO_3 (0,3%) and $MgSO_4$ (0,1%) as a source of mineral salts.

It was mentioned that the production of protease by *Rhodotorula mucilaginosa* CBMAI 1528 depends on the composition of the culture medium and the optimum is obtained in the presence of Glucose and casein Peptone *(Lario et al., 2020).*

The choice of the appropriate fermentation medium is essential for microorganisms, both for growth and for enzyme production. Yeast enzyme production has been significantly improved by the addition of different sources of Carbon and Nitrogen *(Lagzouli et al., 2007).*

For the efficacy of enriched crop media, maximum protease production was found in Candida humícola *(Ray et al., 1992), A.oryzae (García-Gomez, 2008), C. Buinensis (Araujo et al., 2010)* and *Yarrowia lipolytica* (*Hernandez et al., 2011*), is obtained in a culture medium containing fishmeal and soybean meal.

Benkahoul et al., (2017) shows that the best growth of *Aspergillus oryzae* is obtained in the presence of corn-steep liquor, whey and salts. While the best proteolytic activity is observed in the test containing corn-steep liquor, decommissioned dates and salts.

- **Effect of carbon source including starch in crop media**

The effect of different carbon sources (Sucrose, Starch, Glucose, Maltose, Fructose and Xylose) on the protease production of the extreme halophilic strain S2 was investigated and it was found that starch increased proteolytic production. While *Kumar et al., (2016)* reported that the protease activity produced by Bacillus MK 22 is highest in the presence of glucose (*Zemmouri and Zemouri, 2017).*

According to Beata et al., (2021), the highest efficiency of surfactin biosynthesis by *Bacillus subtilis* was achieved using starch as a carbon source. *Singh et al., (2014) and Ghribi and Ellouze (2011) and Das and Mukherjee (2007)* found that the carbon source in synthetic media is mainly glucose, and soluble starch, are preferred to glycerol or hexadecane for the production

of lipopeptide biosurfactants by *Bacillus subtilis* strains.

Seventeen strains of amylolytic yeasts were isolated from traditional baking yeast on a semisynthetic medium containing starch as the sole source of carbon *(Lagzouli et al., 2007). Al-Hussuny et al., (2016)* observed that increased starch concentrations improved the growth of amoeba and may be due to the fact that starch is a good source of nutrients.

According to Lopez et al., (2016*)* maximum protease activity (105 IU/mL) was achieved when using soybean meal as a source of protein and carbon. Soybean meal is also an excellent low-cost substrate for the production of proteases by *Y. lipolytica*. And according to *Sandhya et al., (2005)* the use of wheat bran in the production of *A. oryzae* proteases, resulted in the highest protease activity (31.2 U/mL). *Chellappan et al., (2006) and Sumantha et al., (2005)* found that the addition of casein to the culture medium did not lead to an increase in protease production.

- **Effect of Nitrogen source on protease production**

Several authors have mentioned the importance of yeast extract for the growth of microorganisms and also for the production of proteins (enzymes). Yeast extract is generally used as a source of nitrogen and as a source of vitamins.

Yeast extract is necessary for the production of the extreme halophilic strain S2 protease. Among the nitrogen sources studied, the protease activity of the extreme halophilic bacterial strain S2 (an archaebacteria belonging to the Halobacteriaceae family) is better in the presence of yeast extract followed by peptone *(Zemmouri and Zemouri, 2017).*

Rane et al., (2017), Joshi and Shekhawat (2014) and Das and Mukherjee (2007) showed that yeast extract is used as a nitrogen source by *Bacillus subtilis* strains. According to *Lucas et al., (2020),* yeast extract is generally considered to be the main source of nitrogen and also a key nutrient source in growing media. And according to *Beata et al., (2021),* the highest efficiency of surfactin biosynthesis by *Bacillus subtilis* was achieved by using yeast extract as a nitrogen source.

Sayed et al., (2015) showed that the addition of yeast extract significantly improved the performance of yeast-based MFC (Microbial Fuel Cell), which can be attributed to the role of yeast extract as a growth medium or mediator.

Selby et al., (1975) revealed that the growth of lactic acid bacteria by yeast extract was attributed to the presence of large amounts of free amino acids and peptides, as well as other factors.

According to Kawai and Hu, (2009); Li, (2007) and Chen el al, (2007), yeast extract is generally used as a growth stimulant for microorganisms. Shahriar et al., (1988) showed that it

was essential to obtain a good growth of *B. amyloliquefaciens*. It also shows a maximum influence by the increase of protease production in *Bacillus sp (Prakasham et al 2006)* and *Streptomyces. Sp (Mehte et al., 2006).*

- **Role of peptone and other nitrogen sources**

Rane et al., (2017) and Joshi, Shekhawat (2014), Ozturk et al., (1992) and Das and Mukherjee (2007); show that peptone is used as a nitrogen source for *Bacillus subtilis.*

According to Priatni et al., (2017), peptone was applied as a nitrogen source for bacterial growth. Although the molecular mechanisms of the effects promoting growth in the presence of peptones are not fully understood, the positive effects observed may be due to the diverse amino acid composition of peptones *(Burteau et al., 2003 and Franek et al., 2004).*

Nurdiyana et al., (2015) shows that fish peptone is one of the alternative approaches to the nitrogen source necessary for microbial growth. The need for nitrogen sources differs according to the microorganism, and several studies have used different organic nitrogen sources (simple or complex), and inorganic nitrogen sources. And it has been found that amino acids are better at increasing protease production *(Sharma, 2017).*

Different works *(Ghobadi et al., 2010; Stivanid et al., 2009; Sharma, 2017)* reported that protease production is improved by the combination of peptone and yeast extract.

Among the nitrogen sources (beef extract, tryptone, peptone, glycine and casein) used for the production of protease by Pseudomonas fluores, the maximum production is obtained with peptone. The latter is also a good source of nitrogen for protease production by *Nocardiopsis prosine (Ningthoujam et al., 2009)* and *B. licheniformis* BBRC *(Ghobadi et al., 2010).*

Agrawal et al., (2005) obtained a 12-fold increase in the alkaline protease of *A. oryzae* NCIM 649 grown on wheat bran enriched with soy protein. Vishwanatha et al., (2010) observed stimulation of acid protease synthesis in the presence of 4% soybean meal and casein *by A. oryzae* MTCC 5341, grown in FMS on wheat bran.

According to Boukhalfa (2015*)* the enrichment of tomato waste by soybean meal made the production more economical compared to casein which does not allow to increase the production of the enzyme in a very significant way.

Conclusion

- **General conclusion**

Our work aims to produce acid proteases by *Rhodotorula sp 2* on liquid and cheap media (SmF). A recovery of agro-food waste was possible through two fermentations: the first made on medium based on 5 types of waste (Waste of tomatoes, waste of dates, waste of oranges, bran of Lactoserum) and the second on storm waste only;

For the second fermentation the enrichment of medium was carried out through the use of a statistical method (plan of Plackett and Burman) which allows the selection of factors with significant positive effects on both enzyme production and biomass.

For this, seven factors are tested: Glucose, Sucrose, Yeast extrat, Corn-Steep Liquor, Tween 80, Na_2 CO_3, Mg SO_4. The statistical analysis of the results of the foreground of Plackett and Burman and the modeling of the results by the software Minitab 19 according to a multiple linear regression made it possible to select sucrose and yeast extract.

The results obtained in this thesis show that orange waste used as a base medium is a suitable substrate for the production of acid protease because of their richness in carbohydrates and minerals. They are therefore a good source of Carbon; enrichment according to statistical matrices allowed to select corn steep liquor and yeast extract for the supply of nitrogen source and growth factors.

The results also showed the effectiveness of Plackett and Burman matrices in selecting the variables that intervene during fermentation to increase yield and save time. A large difference was noted between proteolytic activity in the base medium compared to the enriched medium (optimized).

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Appendix

Appendix 1: Transplant and Strain Storage Media

o ***YPGA*** (***Y****east* ***E****xtract* ***P****eptone* ***G****lucose* ***A****gar*)

Used for strain preservation. It contains glucose as a carbon source.

- Agar----------4g
- Yeast extract---------- 5g
- Peptone---------- 10g
- Glucose---------- 20g
- Distilled water---------- 1000ml

The medium is sterilized in an autoclave for 20 minutes at 120°C.

Appendix 2: Transplant and Strain Storage Media

o ***YPMA*** (***Y****east* ***E****xtract* ***P****eptone* ***M****alt* ***A****gar*)

Used for strain preservation. It contains glucose as a carbon source.

- Agar----------4g
- Yeast extract---------- 3g
- Malt extract---------- 3g
- Peptone---------- 5g
- Glucose---------- 10g
- Distilled water---------- 1000ml

The medium is sterilized in an autoclave for 20 minutes at 120°C.

Academic year: 2023-2024	**Presented by: Ammari** Asma. **Hafi** Loubna.

Acid protease production by *Rhodotorula sp* 2 : study of the optimization of the production.

Master's degree in biochemistry.

The aim of the work is a study of the production of a protease by culture of a yeast strain *Rhodotorula sp2* on liquid medium (FML) at different pH (acid, neutral and alkaline). Five substrates are tested: tomato waste, orange waste, date waste, wheat bran and whey with a seeding rate of 10^7 cells at 30°C. Initially we start by determination of concentration of the different substrates tested that gives maximum protease production. The statistical method of one factor at a time (OFAT) showed that the best proteolytic activities are obtained in liquid fermentation based on orange waste at pH 4 (acid protease). In a second step, the statistical plans of Plackett and Burman are respectively used to determine and optimize the factors influencing the production of acid protease. Culture media are prepared using a factorial design of Plackett-Burman N=12 trials and N-1 factors: seven real factors (Glucose, Sucrose, Yeast extract, Corn-steep Liquor, Tween80, Na_2 CO_3, Mg SO_4) and four errors. The results obtained show that the significant factors influencing the enzyme production by the yeast *Rhodotorula sp2* are: sucrose and yeast extract. The optimal values of the variables studied are determined by the response surface methodology namely: sucrose 1% and yeast extract 2%. The validation of the model allowed to obtain an acid protease activity of 17555.55 IU/ml, which is 2 times higher than under non-optimized conditions. These significant results confirm the importance of statistical design in acid protease production.

Keywords: Acid proteases , *Rhodotorula sp*, FML, Tomato waste, Orange waste, Date waste, Wheat bran, Whey, Optimization.

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