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The production of protease from the yeast *Clavispora lusitaniae* and some of it applications

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Acknowledgments and Dedications

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Dedication

This work is wholeheartedly dedicated to my parents whose support and guidance never left me

To my aunt for encouragement and inspiration

To my brothers and sister for their continued help

And especially to my beloved grandma who didn't get to see the fruit of my journey

From: Mosbah Saba

Dedication

With the help of God Allah, I was able to accomplish this work

which I dedicate:

To my dear mother ***Baitiche Fatiha*** *and my father* ***Khallef****, source of life, love and support.*

To my dear brothers, ***Billel, Toufik,*** *and* ***Hichem,*** *source of joy and happiness.*

To all my friends, and my biology colleagues ***Rania*** *and* ***Badr Rihan****.*

Khadidja ♥

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Abbreviations

SSF : Solide State Fermentation.

YPGA: Yeast Extract-Peptone-Gelose-Agar.

TCA: TriChloroacetic Acide.

MCA: Milk Clotting Activity.

T: Temperature.

C°: Celsius degree.

H: Hour.

ml: Milliliters.

SDS: Sodium dodecyl-sulfate.

PAGE: Poly Acrylamide Gel Electrophoresis.

pH: Hydrogen potential.

BSA: Bovine Serum Albumin.

µl: Micro-liters.

µg: Micro-grams.

KDa: Kilo Dalton.

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Introduction

Pollution of the environment by industrial household waste (food and other waste and leftovers) and by chemicals is constantly increasing, altering our biotope. Recycling and reclaiming this waste is one way of reducing its harmful impact on our planet. Various food wastes can be promising alternatives, used as a carbon source and as a support for microorganisms to produce value-added metabolites, namely microbial enzymes. The latter are key ecological alternatives to standard chemicals.

Fungal enzymes have found their way into a variety of industries, and are one of the key alternatives most in demand.

Consequently, any substantial reduction in the cost of enzyme production will provide a positive stimulus for enzyme commercialization. Proteases are one of the largest groups of industrial enzymes, accounting for almost 60% of total enzyme sales (Savitha et al., 2011).

Proteases are mainly used in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, digestive product production, and some medical treatments of inflammation and virulent wounds (Paranthaman et al., 2009). In addition, they are used in silk degumming, the composition of ointments and soft-gel medicinal formulas helps remove silver from used X-ray film, waste management in the poultry and leather industries, and as a detergent additive to remove protein-based stains (Mukhtar, 2016a). They are also used to improve the digestibility and sensory quality of foods, as well as providing health benefits by reducing allergenic compounds (Tavano, 2013) and modifying dough properties in baking (Owen, 2011).

Solid substrate fermentation (SSF) was chosen for the present research because it has been reported to be much more productive than submerged fermentation (Paranthaman et al., 2009). From an economic point of view, SSF offers many advantages, including higher volumetric productivity, the use of simpler machinery, the use of inexpensive substrates, simpler downstream processing, and lower energy requirements compared with submerged fermentation (Arora et al., 2018).

As far as the enzyme market is concerned, Algeria lags far behind in terms of enzyme use and production, relying mainly on imports rather than local production, resulting in significant economic losses that could easily be avoided.

When it comes to the enzyme market Algeria lags well behind in terms of both enzyme employment and production, depending mainly on imports rather than local production which results in significant economic losses that could be easily avoided.

And so, building on precedent research results we chose the locally available strain *Clavispora lusitaniae* taking advantage of one of the most wasted resources of the country bread and tomatoes leftovers as a substrate, and proceeded with solid-state fermentation to produce protease, partially purified it, and then test it out to investigate its industrial potential. To advocate for a positive change, we wrote this study with the following objectives in mind:

- Producing protease from a locally available strain *Clavispora lusitaniae*
- Providing a new opportunity to recycle wasted bread and tomatoes
- Introducing a few prospective protease industrial applications
- Further push the development of the enzyme market in Algeria

The study was conducted in two main parts.

The first part includes a literature review on the main elements of the study, from general information on the yeast *Clavispora lusitaniae* and the enzyme protease to their contribution to the biotechnology industry.

The second part is devoted to the methods used to achieve the targeted objectives, the results obtained, and its discussion, all coming to an end with a general conclusion.

Literature review

Chapter1 Yeast

1. Generalities

Yeasts are eukaryotic, unicellular micro-fungi. First observed by A. Van Leewenhoek in 1680. The term "yeast" in English and many other languages takes the meanings of "foam" and "to rise" directly reflecting its role as a fermentation agent (Kurtzman et al., 2011) such microorganisms have been used throughout human history in the fabrication of bread, dairy products, wine, and other alcoholic beverages (Labrecque, 2003). Nowadays, Yeasts are regarded as a model organism due to its low food requirements and rapid development and so in addition to its traditional usages it's employed to produce metabolites (lipids, organic acids, polysaccharides, vitamins) (Labrecque, 2003)and especially commercial enzymes such as amylase, cellulase, lactase and protease (Meyer et al., 1988; Scriban, 1999)

2. Morphology and structure

Yeasts are typically white (rarely pink or red), spherical or ovoid in shape, with cells ranging in size from 5 to 20µ.(Barnett et al., 2000; Guiraud, 2003). These aspects and dimensions are usually determined by the species type, culture conditions, and cell age.(Scherr and Weaver, 1953)

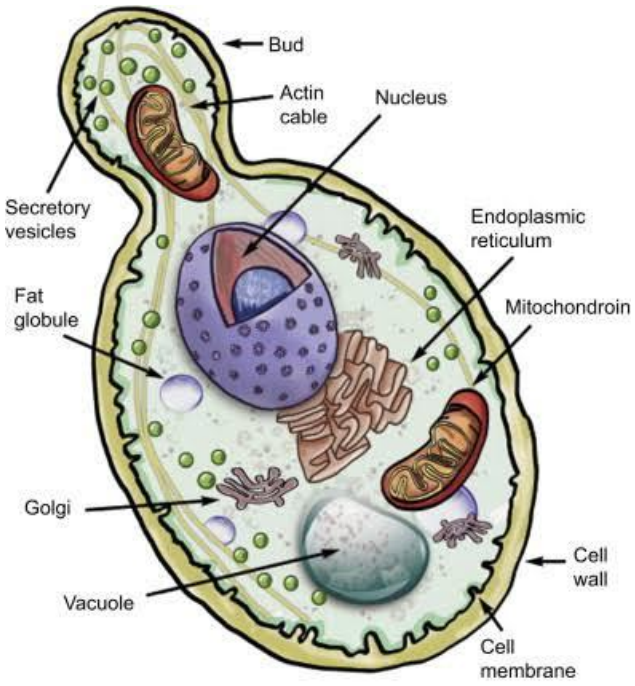

Figure 1: A diagram of a yeast cell structure *(Speers and Forbes, 2015)*

3. Habitat

Yeasts are ubiquitous microorganisms that are compatible with a variety of environments including: air (Belhomsa et al., 2017; Péter et al., 2017), soil (Merabti, 2006), fresh and deep water (Droop and Wood, 1968; Meyer et al., 1988; Scriban, 1999; Stevenson et al., 1973). Some yeasts have been found in the extremely cold polar region (Baeza et al., 2019). Others live mainly on plants rich in sugars, especially fruits (Tsegaye and Tefera, 2018) like grapes (Oteng-Gyang, 1984), mango (Moussa and Andrianarisoa, 2015). Also, food products such as yogurt (Lopandic et al., 2006), cheese (Binetti et al., 2013), honey (Jiang et al., 2018).

4. Reproduction

Depending on the environmental condition yeasts can go through two types of reproduction

4.1 Asexual reproduction

The majority of yeasts reproduce through budding (**Figure 2**). A bud is a tiny protrusion that extends from the parent cell and produces a new individual when it reaches maturity.

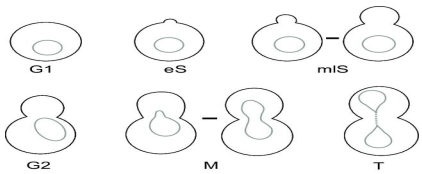

Figure 2: A diagram of a budding yeast cell (Neumann and Hediger, 2006)

The other way, though not as common, is through binary fission (**Figure 3**), in which the parent cell gets elongated and its nucleus divides and gradually separates near the middle into two daughter cells. (Larpent, 1991)

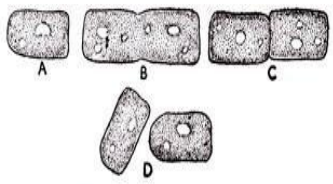

Figure 3: A diagram of a yeast in binary fission. *(Mehak, n.d.)*

4.2 Sexual reproduction

Different-gender haploid yeast cells fuse to form a diploid cell that goes through mitosis to produce a population of diploid yeast cells (**Figure 4**).

In the case of adverse conditions, diploid yeast cells produce haploid endospores, which mature into haploid yeast cells after germination (François et al., 2001).

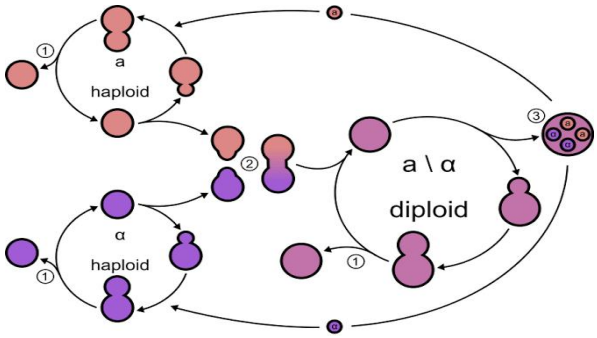

Figure 4: Yeast reproduction diagram *(Lakna, n.d.)*

5. Nutritional needs and physico-chemical requirements

5.1 Nutritional needs

5.1.1 Carbon sources

For the well-development of the yeast, carbon is one of the essential requirements, accounting for 50% of its total dry weight (Toumi, 2018). It is used as both a source of energy and a building component for its cellular constituents.(Walker, 2009)

For yeasts, monosaccharides such as glucose, fructose, and mannose are the most effective sources(Pol, 1997); however, saccharides, polyols, alcohols (ethanol, methanol, and glycerol), polysaccharides (soluble starch, pectin), and organic acids (lactic acid, citric acid, tartaric acid, malic acid, succinic acid, and others) may be utilized as well by yeast. (Brou and Paul, 2018; Dakhmouche, 2016; Moussa and Andrianarisoa, 2015; Ponomarova et al., 2017)

5.1.2 Nitrogen sources

Yeast uses nitrogen to stimulate its growth by facilitating the synthesis of proteins, nucleic acids, and vitamins(Bourgeois and Leveau, 1980; Toumi, 2018). It can draw nitrogen from a variety of sources, be they organic (such as peptone, yeast extract, glutamine, aspartic acid, purines, and pyrimidine bases) (Deak, 2006; Gobert et al., 2017; Walker, 2009) or inorganic such as ammonium salts (Brou and Paul, 2018), nitrates (Bourgeois and Larpent, 1996).

5.1.3 Mineral elements and growth factors

Mineral elements and growth factors are very important for the yeast metabolism and overall growth on the condition of low concentration(Larpent-Gourgaud and Sanglier, 1992),

when said concentration goes beyond a certain level, it leads to the denaturation of enzymes and a disturbance of cell morphology and physiology (Blom et al., 2000).

Mineral elements that contain K^+, Na^+, P^{2+}, Mg^{2+} ...etc, are components of metalloenzymes involved in metabolic pathways (Brou and Paul, 2018) while growth factors such as vitamins (biotin, pantothenic acid, myoinositol, nicotinic acid and thiamine) are involved in the structure of coenzymes of the yeast metabolic enzymes, they therefore act on cellular activity and division (Brou and Paul, 2018).

5.2 Physico-chemical requirements

5.2.1 Temperature

Due to the large influence of temperature on biologic reactions, each organism has an optimal growth temperature, and yeasts are no different. Taking into account its diversity and various habitats, pinpointing a strict temperature interval that suits all is impossible. In general, yeast's culture temperature ranges between 35 and 45 °C to ensure adequate growth (Buzzini et al., 2018), for thermophilic yeasts. Minimum growth can be between 20 °C and 50 °C (Liszkowska and Berlowska, 2021). Psychrophilic yeasts, on the other hand, reach their maximum growth at temperatures between 5 °C and 20 °C 4, and around 0 and 50 °C for mesophilic yeasts development. (Dakhmouche, 2016).

5.2.2 pH

Yeasts can tolerate pH levels ranging from 2.4 to 8.6. Their optimum growth is observed at pH levels ranging from 4 to 6.5. (Chniti, 2015).

5.2.3 Oxygen

All yeasts grow in the presence of oxygen; there are no strict anaerobes; some are strict aerobes, while others are facultative aerobes. (Bourgeois and Leveau, 1980).

5.2.4 Osmotic pressure and water activity

The effects of osmotic pressure on the development of the organism vary from one yeast to another. Most yeasts cannot grow in water activity of less than 0.90, while some can tolerate higher osmotic pressures corresponding to a water activity of 0.60, but they have a slow metabolism. These yeasts are referred to as "xerotolerant" because they can produce osmoprotectants (betaine and glycerol).(Bourgeois and Leveau, 1980; Larpent-Gourgaud and Sanglier, 1992).

6. *Clavispora lusitaniae*

Clavispora lusitaniae is a yeast of irregular shape that tend to range from round to elongated reproduce by multilateral budding and has no known ecological niche It can be isolated from a variety of substrates, including soils, waters, plants, and the gastrointestinal tracts of a wide range of animals, including birds, mammals, and humans.

C. lusitaniae can be pathogenic for the immunocompromised and newborn hosts and is responsible for approximately 19.3% of fungemia cases in cancer patients, and about 1% of invasive candidiasis, particularly in pediatric and hematology-oncology patients.

6.1 Taxonomy

Kingdom: Fungi

Subkingdom: Dikarya

Phylum: *Ascomycota*

Subphylum: *Saccharomycotina*

Class: *Saccharomycetes*

Order: *Saccharomycetales*

Family: *Metschnikowiaceae*

Genus: *Clavispora*

Species: *lusitaniae* (Butler et al., 2009)

Chapter2 Protease

1. Generalities

Proteases, also known as proteinases or peptidases, are very complex enzyme groups produced extracellularly or intracellularly and classified under the hydrolases class (EC 3.4.21–24.x).(Kumar et al., 2008a; Qureshi et al., 2011).

As the name implies, they hydrolyze proteins at very specific sites by splitting the peptide bond between two amino acids in a peptide chain. (Kumar et al., 2008b).

They are involved in many biological processes, including metabolism regulation, gene expression, and the hydrolysis of large proteins into smaller molecules for transport and metabolism (Rao et al., 1998) .

They are currently regarded as the most important commercial enzymes, accounting for more than 65% of the global enzyme market. (Ibrahim et al., 2015)

2. Classification

proteases are classified according to several major criteria based on cellular localization, the length of the polypeptide chain, the nature of the catalytic site, and according to the optimum pH activity.

2.1 According to cell location

Proteases are divided into two groups based on their location within the cell: intracellular and extracellular proteases, with the latter being more intriguing for industrial applications because they are easier to extract. (Drouin, 2005; Rao et al., 1998)

2.2 According to the length of the polypeptide chain

This classification is based on the protein's final form after protease action, which results in two types: those that divide the protein molecule into polypeptide fragments and those that hydrolyze the polypeptides and transform them into free amino acids. (Colwell, 2012; Frazier and Westhoff, 1988).

2.3 According to the catalytic site on the substrate

Proteases are classified into two main groups based on action site: endopeptidases and exopeptidases, both are further subdivided into subclasses (Rao et al., 1998) (**Figure 5**).

Endopeptidases: (break the covalent bond inside the chain further from the termini). They are very little used in industry.

Exopeptidases: (break at the NH_2 and COOH ends of the targeted protein) and are the most used in industry(López-Otín and Bond, 2008)

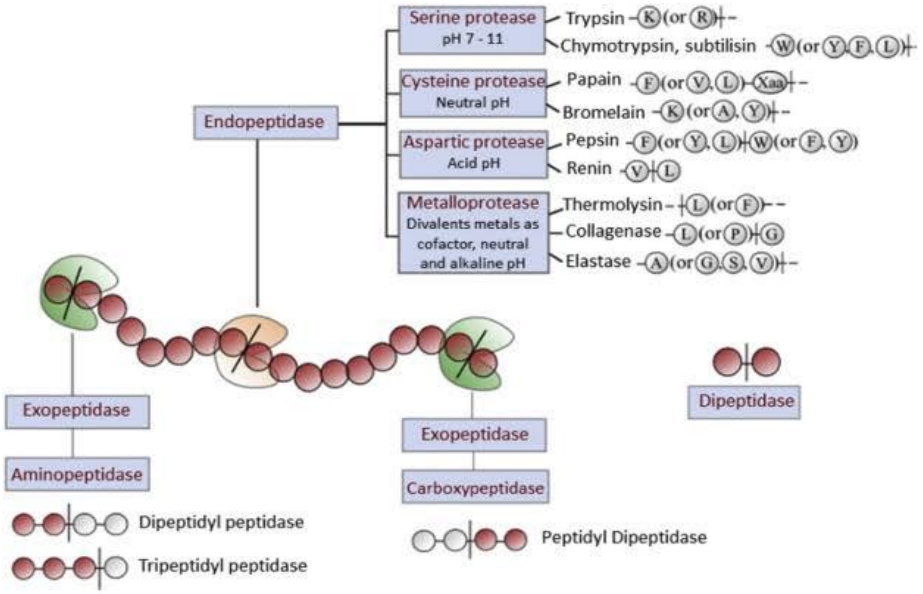

Figure 5: Classification of protease based on their action at and away from the termini.

(Fleuri et al., 2022)

2.4 According to the optimal pH of the activity

Proteolytic enzymes are classified into three groups according to their optimum pH: acidic, neutral and alkaline (Razzaq et al., 2019) **(Table 1).**

The alkaline protease producers are distributed in water, soil, and highly alkaline conditions between 9 and 11 while the optimum pH of acidic proteases is 3–4 with a molecular weight of 30–45 kDa as for neutral proteases they are active at a neutral or weakly acidic or weakly alkaline pH. Mostly neutral proteases belong to the genus *Bacillus* and with a relatively low thermotolerance ranging from pH 5 to 8.

Table 1: A comparison of different proteases

Type of proteases	pH range	Uses of proteases	Classification	Sources	References
Alkaline	9–11	Detergent and leather industry	Serine proteases, subtilisin Carlsberg and subtilisin novo	Mostly produced by bacterial species, such as A. *salinivibrio sp.* strain AF-2004, marine shipworms, *Cryptococcus aureus*, mushrooms, *Bacillus sp.*	(Dodia et al., 2008; Miyaji et al., 2006; Patil and Chaudhari, 2009; Simkhada et al., 2010; Soroor et al., 2009; Vadlamani and Parcha, 2011)
Acidic	3.8–5.6	Soy sauce, protein hydrolysate, digestive aids and in production of seasoning material, clearing beer and fruit juice, improving texture of flour paste and tendering the fibril muscle	Aspartic proteases, pepsin (A1), retropepsin (A2) and enzymes from Para retroviruses (A3)	Mostly produced by fungal species, such as *A. niger*, *A. oryzae*, *A. awamori*, *A. fumigatus*, and *A. saitoi.*	(Pushpam et al., 2011; Sielecki et al., 1991; Steele et al., 1992; Zhang et al., 2010)
Neutral	5–8	Food industry, brewing industry	Neutrase, thermolysin	Genus *Bacillus*	(Sodek and Hofmann, 1970)

3. Protease sources

Because proteases serve vital physiological functions in several biological processes, they are found in all living beings, whether animals, plants, or microorganisms.(Raimi et al., 2010).

3.1 Animal proteases

The pancreas synthesizes a major portion of animal proteases in the form of a precursor that can be activated autocatalytically under well-defined conditions.(Lakba, 2015).

Animal-derived proteases, such as trypsin and chymotrypsin, are employed in food (rennet) and medicine. (Rao et al., 1998)

3.2 Plant proteases

Many studies have been conducted on plants in attempt to isolate proteolytic enzymes. Several factors influence plant-based enzyme production, including the availability of land for cultivation and the suitability of climatic conditions for growth. Furthermore, the production of proteases from plants is time-consuming.(Rao et al., 1998).

Plant proteases isolated from papaya (*Carica papaya*), fig tree (*Ficus glabrata*), pineapple (*Ananas comosus*), and melon sarcocarp (*Cucumus melo*) are especially well-known. They are most commonly used in the food and pharmaceutical industries.(Mahajan and Badgujar, 2010).

3.3 Microbial proteases

Produced by a wide variety of bacteria, molds, and yeasts proteases of microbial origin offer several advantages over those of animal or plant origin, including high growth rates leading to short fermentation times and their ability to secrete proteins into the extracellular environment.(Devi, 2008; Dos Santos Aguilar and Sato, 2018)

3.3.1 Bacterial proteases

Proteases derived from bacterial strains are widely used in the biotechnology industry for a variety of applications. One of the most commonly used strains is *Bacillus sp* produces subtilase extracellularly into the environment, facilitating its purification its stability (Loudjani, 2016; Owen, 2011)

Other proteases from different bacterial strains, such as *Pseudomonas, Streptomyces, Enterococcus, Listeria, Thermus, Oenococcus, Chrysobacterium,* and *Microbacterium*, are also available in the biocatalysis market.(Anisha et al., 2008; Mahajan and Badgujar, 2010)

3.3.2 Molds and fungal proteases

Aspergillus, Penicillium, Trichoderma, Mucor, Rhizopus, Geotrichum, Fusarium, Rhizomucor, Endothia, and other fungi and molds are utilized in the manufacturing of industrial protease. They have the benefit of isolating mycelium by simple filtration and can be easily manufactured through a solid-state fermentation technique.(Frazier and Westhoff, 1988; Jisha et al., 2013)

The proteases produced are employed in baking, in the food and feed industry, in laundry detergents, in the tannery industry and in the pharmaceutical industry (Ul-Haq and Mukhtar, 2009)

3.3.3 Yeast proteases

Yeasts combine a microorganism's ease of genetic manipulation and fermentation with the ability to produce and change foreign proteins in accordance with a general eukaryotic scheme. Their quick growth and microbiological safety have a significant impact, particularly in large-scale industrial protein synthesis. As a result, various yeast species, including *Saccharomyces, Rhodotorula, Candida*, and *Debaryomyces*, are used in the manufacture of proteolytic enzymes.(Boiron and Périlleux, 1996; Idiris et al., 2010).

Chapter3 Production

1. Proteases producing microorganisms

Protease can be obtained from almost any living organism. For industrial production, microorganisms such as bacteria, fungi and yeasts are generally used for their ease of cultivation, low cost and high yield on a large scale, in the presence of the appropriate favorable conditions (Rao et al., 1998).

1.1 Conditions effect on proteases production

Microbial protease production, in general, is influenced by several variety of conditions as culture mediums, temperature and pH specific for each species.

1.1.1 Protease producing yeasts

The rapid growth and high-density fermentation of yeast in a chemically defined medium have a significant impact, especially in the industrial production of proteins (Porro et al., 2005).

This has led to an expansion of studies on improving protein production using yeast as cell factories, and several therapeutic proteins are currently being produced using yeast (Rader, 2008; Schmidt, 2004).

Despite reports regarding yeast proteases, relatively little is known about the properties of extracellular proteases. Therefore, the protease properties of some yeast species were studied according to medium composition, pH, and temperature (Kim, 2019a). **(Table 2)**

Table 2: Growing conditions of different protease producing yeasts

Strain	pH	T	Substrate	Reference
Citeromyces matritensis	5	30	Sodium Casein and glucose.	(Rodarte et al., 2011)
Aureobasidium pullulans	9	45	—	(Ma et al., 2007a)
Pichia anomala CO-1	7	30	—	(Kim, 2019b)

Candida humicola	1-5	37	—	(Ray et al., 1992)

1.1.2 Protease producing bacteria

The majority of neutral and alkaline commercial proteases are produced by the *Bacillus* genus. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have a relatively low heat tolerance. Whereas alkaline bacterial proteases are characterized by their higher activity at an alkaline pH, for example, pH 10, and their broad substrate specificity (Rao et al., 1998). **(Table3)**

Table 3: Growing conditions of different protease producing bacteria.

Strain	pH	T°C	Substrat	Reference
Bacillus subtilis	5-8	25	Feather culture medium	(Suh and Lee, 2001)
Chryseobacterium sp.	8	30	Chicken feathers	(Riffel et al., 2003)
Bacillus pumilus keratinase	8	37	Chicken feathers	(El-Refai et al., 2005)
Streptomyces thermoviolaceus	8	55	Muscle, collagen, hair, nail, feathers.	(Chitte et al., 1999)
Thermoanaerobacter keratinophilus	8	85	Anaerobic complex medium with chicken feathers, merino wool, or human hair	(Sabine and Garabed, 2001)
B. subtilis	8	50	Casein.	(Yang et al., 2000)

1.1.3 Proteases producing fungi

Fungi produce a wider variety of enzymes than bacteria. For example, *Aspergillus oryzae* produces acid, neutral, and alkaline proteases. The fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity. Fungal enzymes can be conveniently produced in a solid-state fermentation process. Fungal acid proteases have an optimal pH between 4 and 4.5 and are stable between pH 2.5 and 6.0. Fungal-neutral proteases are metalloproteases that are active at pH 7.0. (Rao et al., 1998) **(Table 4)**

Table 4: Growing conditions of different protease producing fungi.

Strain	Fermentation	pH	T	Total/specific activity (U)	Substrat	Reference
Cordyceps militaris	SmF	8.5 - 12	25	818.7	Colloidal chitin.	(Hattori et al., 2005; Jin et al., 2021; Kumar das, 2010)
Thermomyces lanuginosus	SmF	5	70	12.8	Casein,glucose, yeast extract.	(Li and Yang, 1997; Pathak and Rathod, 2018; Zou et al., 2020)
Aspergillus oryzae IAM2704	SmF	7	30	1550	Casein, glucose.	(Jin et al., 2021; Katayama et al., 2016; Ogawa et al., 1995)
Aspergillus oryzae MTCC 5341	SSF	3-4	55	43.658	Wheat bran.	(Jin et al., 2021; Katayama et al., 2016; Vishwanatha et al., 2009)
Aspergillus oryzae NCIM 649	SSF	7	36	6301	Wheat bran, soy protein.	(Agrawal et al., 2005; Jin et al., 2021; Katayama et al., 2016)

Chapter4 Application

Proteases are playing an important role in industries due to their wide application in leather and detergent, food and pharmaceutical industries and also in bio-remediation processes (Ben Rebah and Miled, 2013).

Bacterial proteases are produced in large scale due to their high stability, specificity and activity under a wide range of physical conditions (Ningthoujam and Kshetri, 2010).

More than 60% of the worldwide production of industrial enzymes are proteolytic enzymes, Among these 35% is comprised of alkaline proteases (Guangrong et al., 2008), which are extensively used in a wide range of industries such as food, pharmaceutical, detergent, cheese making, brewing, photography, baking, meat tenderization ,cosmetics and leather (Dias et al., 2008; Synowiecki, 2010) **(Table 5).**

Table 5: Different proteases applications.

Source	Protease	Application	Reference
Stenotrophomonas sp.	alkaline protease	Suitable for detergent and textile industry	(Irame et al., 2012)
Serratia marcescens	Metallo-protease	Detergent additive for cleaning purposes	(Tariq, 2011)
Pseudoalteromonas arctica	subtilisin-like protease	Suitable for cold-active laundry or dish washing purposes	(Park et al., 2018)
Pseudoalteromonas sp.	serine protease	Applicable in low-temperature food processing	(Wang et al., 2008)
Pseudoalteromonas sp.	serine protease	Improves the taste of refrigerated meat	(He et al., 2004)
Pseudomonas aeruginosa	alkaline protease	Cold washing detergent enzyme	(Hao and Sun, 2015)
Planococcus sp.	serine protease	Detergent additive for cold washing	(Chen et al., 2018)

Penicillin nalgiovense	alkaline protease	Suitable for meat ripening purposes	(Papagianni and Sergelidis, 2014)
Pedobacter cryoconitis	Metalo-protease	Bioremediation of wastewater in cold conditions	(Margesin et al., 2005)
Flavobacterium limicola	Cold-active protease	Primary mineralization of organic polymers in freshwater sediments	(Tamaki et al., 2003)
Enterococcus faecalis	Metalloprotease	Improves the stability and solubility of health foods	(Root-Gutteridge and Chatterjee, 2009)
Chryseobacterium sp.	Serine protease	Applicable in meat and other food processing units	(Mageswari et al., 2017)
Bacillus sp.	Metalloprotease	Environmentally friendly feed additive to improve the production performance of farm animals	(Park, 2011)
Bacillus sp.	Metalloprotease	Detergent additive for cold-washing	(Furhan et al., 2019)
Arsukibacterium ikkense	Cold-active protease	Applicable in dairy products and other functional foods	(De Gobba et al., 2014)
Acinetobacter sp.	Serine protease	Suitable for detergent formulations	(Salwan and Kasana, 2012)

1. Food industry

Proteases are employed in the production of wine, bread, cheese, and butter. In the dairy industry, primarily attacking peptide bonds in cheese. Acid protease is used to produce ethanol, which can be used as a nutrition source for yeast (naveed et al., 2020).

Certain fungal strains, such as *Mucor michei* , are employed to manufacture acidic proteases that can be used to replace animal renin in cheese production.

Alkaline proteases are used in soy sauce and enzymatic reactions that lead to the production of a high-quality protein called hydrolysates .(Sharma et al., 2019)

Fungal proteases are utilized in the fruit juice and beverage industries to degrade complicated substances by acting on peptide links between proteins .**(Figure 6).**

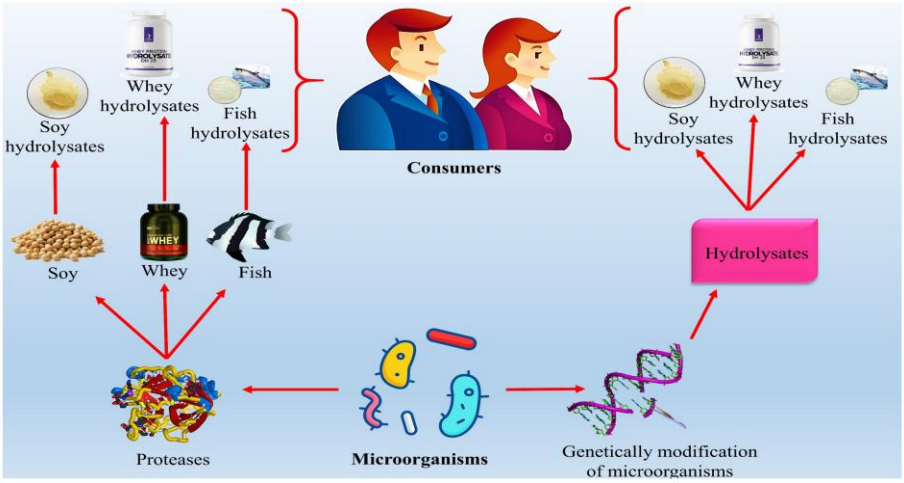

Figure 6: Applications of fungal proteases in the food industry. (Naeem et al., 2022)

1.1 Baking industry

Wheat flour is an important ingredient in baking. It contains gluten, an insoluble protein that controls the qualities of bread dough.

Endo and exoproteinases from *Aspergillus oryzae* have been utilized to alter wheat gluten via restricted proteolysis. Enzymatic treatment of the dough makes it easier to handle, lowers mixing time, and results in larger loaf volumes.

Bacterial protease are employed to increase the dough's flexibility and strength (Rao et al., 1998) **(Figure 7).**

Figure 7: Protease can boost bakery sector . **(Web1).**

1.2 Manufacture of soy products

Soybeans are a high-protein food source, Protease have been used to make soy them and other soy products since ancient times.

Fungal alkaline and neutral protease have an important function in the production of soy sauce, Soy proteins treated with alcalase at pH 8 give soluble hydrolysates with great solubility, good protein output, and minimal bitterness. The hydrolysate is utilized in the production of protein-fortified soft beverages as well as the formulation of dietetic feeds (Rao et al., 1998) **(Figure 8).**

Figure 8: Applications of enzymes within soybeans. **(Web2).**

1.3 Synthesis of aspartame

The food and drug administration has approved using aspartame as a non-calorific artificial sweetener. Aspartame is a di-peptide comprising L-aspartic acid and the L-

phenylalanine methyl ester, the sweetness of aspartame is caused by the L configuration of the two amino acids.

Although proteases are commonly thought of as hydrolytic enzymes, they catalyze the reverse reaction under certain kinetically regulated conditions. For the enzymatic production of aspartame, an immobilized preparation of thermolysin from *Bacillus thermoprotyolyticus* is utilized. The two biggest industrial producers of aspartame are Toya Soda (Japan) and DSM (the Netherlands) (Rao et al., 1998) .

2. Protease as therapeutic

2.1 Protease as a New Therapeutic Strategy for Celiac

Celiac disease is an autoimmune digestive disorder that affects the small intestine due to poor digestion of gluten-based toxic peptides such as gliadin, resulting in gut inflammation **(Figure 9)**.

When gluten is hydrolyzed by normal digestive enzymes, immunotoxic gliadin peptides are generated. Gluten processing produces gliadin peptides high in proline and glutamine residues, and a 33-mer gliadin peptide has been linked to the onset of the inflammatory cascade in celiac disease (Shan et al., 2002).

Proteases that break down gluten peptides may be employed to treat celiac disease (Sollid, 2002). As of Lately, several protease-based medicines combined with a gluten-free diet and a healthy diet plan have effectively treated celiac disease (Green et al., 2015).

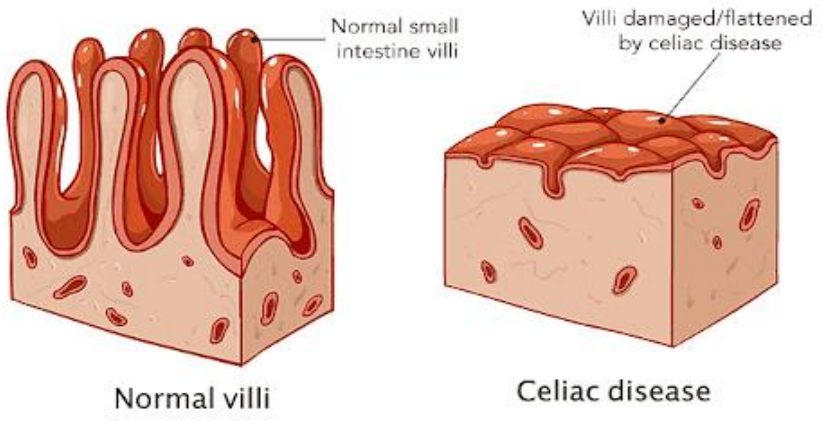

Figure 9: Lining of the small intestine **(Web3).**

2.2 Protease as a new therapeutic strategy for colorectal cancer

Colorectal cancer is the most common type of cancer and has a high prevalence rate in developed countries due to an unhealthy diet. Although several drugs have been used to treat it in recent decades, the disease is still fatal in its advanced stages.

Proteases have been utilized to inhibit tumor formation at the cellular level.

A protease-based therapy for colorectal cancer is a ubiquitin-proteasome system(López-Otín and Matrisian, 2007). Which is a multiplex protease enzyme system necessary for cell survival that induces the destruction of misfolded proteins. It is also important in controlling the turnover of aberrant proteins or short-lived regulatory proteins that are engaged in vital physiological processes such as cell death and signaling pathways, apoptosis, metastasis, and cell proliferation (Kwon and Ciechanover, 2017).

To create the ubiquitin linkage, protein substrates are primarily attached to the chain of the protein ubiquitin in the presence of ubiquitin-activating enzymes or E1, ubiquitin-conjugating enzymes or E2, and ubiquitin ligases or E3.

The polymerized ubiquitin chain serves as a signal to convey the target proteins to the proteasome, where they are broken down by proteolysis (Liu et al., 2015).

3. Bioremediation and Waste management

Poultry and leather industry wastes are rich in keratin that is densely packed and stabilized by hydrogen, hydrophobic interactions and di-sulfide bonds (Steinmann et al., 2002).

These keratin wastes are degraded by chemical and mechanical hydrolysis which is not eco-friendly. Enzymatic degradation by using alkaline proteases is better method (Kudrya and Simonenko, 1994).

Bacillus species is the most widely reported bacterial source of keratinases for feather degradation. Other reported bacterial sources of keratinases are *Pseudomonas sp. MS21, Microbacterium sp., Chryseobacterium sp. and Streptomyces sp.* Fungal keratinases obtained from *Aspergillus oryzae, Chrysosporium indicum, Trichophyton mentagrophytes, Microsporum sp., Trichophyton sp., Aspergillus terreus, Scopulariopsis sp., Fusarium oxysporum* have also been studied towards the degradation of keratin (Sharma et al., 2010)**.**

4. Proteases in Silver Recovery

In general, X-ray and photographic films contain 2% silver incorporated in the gelatin layer. This silver can be recovered using a variety of methods. Formerly employed methods retrieved silver recovery by burning X-ray films, which causes environmental problems.

The carbon monoxide explosion also increased the risk of respiratory infections in nearby areas. As a result, traditional silver recovery methods are ineffective, and an urgently advanced approach is required to reduce environmental and safety concerns (Qamar et al., 2020).

Alkaline proteases have been used successfully for silver recovery while posing no environmental risks **(Figure 10)**.

Proteases from the *Bacillus* species can catalyze the hydrolysis of the gelatin layer with optimum silver retrieval while avoiding damage from a recyclable polyester base film(Patil, 2012).

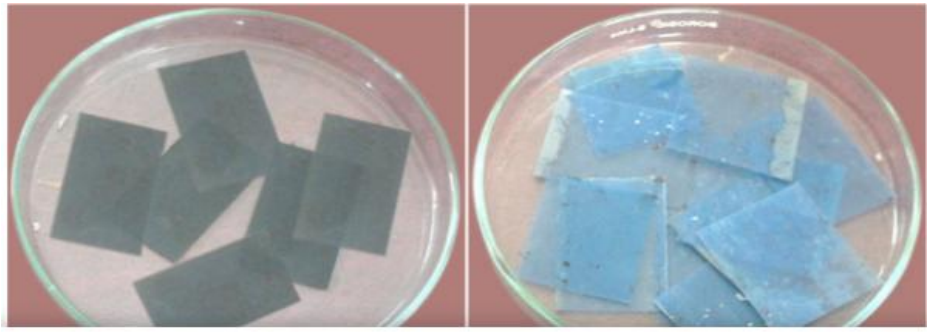

Figure 10: X-ray film after enzymatic hydrolysis of gelatin-silver layer. (Lakshmi and Hemalatha, 2016).

5. Proteases in Silk-Degumming

Sericin, a fibrous protein that surrounds silk fibers and gives them a rough texture, protects them. To organize the structure of silk fibers, various conventional methods for removing sericin have been used. (Pierre and Jérome, 2008)

The high-cost machinery required to extract the sericin makes these procedures pricey.

Proteases have been used to degum silk in order to remove sericin while maintaining fiber structure. Prior to using traditional procedures, alkaline protease produced from *Bacillus sp.*, RGR-14 is used to degum silk. (Nakpathom et al., 2009).

6. Detergent industry

Microbial cold-adapted proteases with detergents are more effective during cold washing than enzyme-free detergents. Moreover, cold-adapted alkaline proteases have demonstrated excellent stability in commercial surfactants and bleaches (Hao and Sun, 2015).

alkaline proteases from *Bacillus cereus, Bacillus pumilus strain CBS, Streptomyces sp. strain AB1,Bacillus licheniformis, Aspergillus flavus, Aspergillus niger, Bacillus brevis, Bacillus subtilis AG-1* have exhibited excellent detergent compatibility in the presence of certain stabilizers such $CaCl_2$ and glycine (Abou-Elela et al., 2011).

Proteases combined with commercial detergents removed proteinous materials from garments stained with chocolate, tea, blood, egg yolk, grass, and other substances at considerably lower temperatures than enzyme-free detergents. These qualities make them detergent additives for the laundry and dishwashing sectors (Sarmiento et al., 2015)**.**

7. Leather Industry

Proteases are essential in the treatment of raw leather in tanneries **(Figure 11),** Soaking includes removing blood, feces, and filth from hides as well as making certain structural modifications. The use of alkaline serine proteases is the most effective leather treatment. Alkaline proteases are important in the solubilization of albumin and globulin, the opening of constricted fibrous proteins, and the washing of debris and excess fat during the soaking stage (Mukhtar, 2016b).

The lime-sulfide procedure is the traditional method for depilation that involves the use of sodium sulfide and hydrated lime. On the flesh side of the skin, a paste of sodium sulfide and hydrated lime is applied. The goal of this therapy is to remove hair through mucoid hydrolysis, collagen fiber swelling, and elastin digestion. This process is now objectionable worldwide because of the massive release of dangerous chemical compounds (Mukhtar, 2016b).

Proteases, which are usually generated by bacteria and are stable in alkaline environments, have become increasingly popular for depilation and skin opening. Enzymatically aided dehairing techniques enhance the leather's surface area and make washing and dyeing easy. Proteases with a high pH activity can penetrate the skin more easily. *Bacillus subtilis* produced proteases with keratinolytic activity that replace sodium sulfide in dehairing process of leather industry .and microbial proteases have replaced the use of trypsin, as they are more economical.(Mukhtar, 2016b).

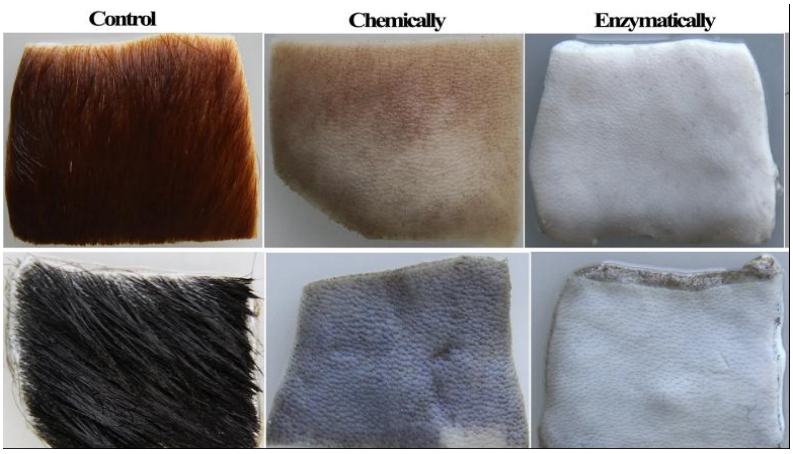

Figure 11: Enzymatic dehairing in the leather industry. (Zhou et al., 2018).

Materials and methods

1. Production stage

1.1 Yeast strain

The yeast used for the production of the protease is the strain *Clavispora lusitaniae CBS 6936 T* isolated from potato peels. It is kept in themycotheque of the Enzymatic Engineering and Application laboratory.

1.2 Reactivation of yeast strain

The reactivation of the yeast is carried out on a new nutrient culture medium YPGA **(Appendix 1)** inoculated by the streak method in petri dishes using a Pasteur pipette under aseptic conditions. The plates are then incubated for 48 hours at 30°C in an incubator **(Figure 12).**

Figure 12: Tools used in the reactivation of the strain.

1.3 Preparation of the cell suspension

A quantity of YPGA medium is poured into a petri dishes and then seeded with *Clavispora lusitaniae.* After incubation for 48 hours at 30°C, a quantity of sterile distilled water (or physiological water) is added, and the cells are homogenized by manual shaking. Recover the stock yeast solution to carry out the cell counting and inoculation of the media. The stock solution is the inoculum and should be kept in the refrigerator

1.4 Cell counting

The number of cells was estimated by counting directly from a drop of the yeast suspension using a Thomas cell under an optical microscope magnification of (× 40).

Thomas cell is engraved with a special grid making it possible to count within, each yeast cell one by one, after determining the number per unit using the following rule it's possible to estimate the concentration of cells per milliliter.

$$\text{total cell count} = \frac{\text{total cell count} \times \text{dilution} \,.\, 10^3}{\text{number of tiles} \times 0.00025}$$

1.5 Fermentation substrate

the media is based on tomato waste and the remains of the bread; because these substrates are rich in nutrients necessary for the development of yeasts and also for the production of enzymes.

The drained tomato waste and the remains of the bread recovered are dried in the open air (25– 30°C). Then crushed to have into powder **(Figure 13).**

Figure 13: Erlenmeyers after adding the substrate.

1.6 Fermentation media

Fermentation is carried out in a set of 8 Erlenmeyer flasks of 250ml volume containing a 10g mixture of two types of waste serving as substrates (5g crushed bread scraps, 5g powdered tomato peelings). 9.7ml of buffer are added to obtain a moisture content of 96.82%. The contents of each flask are then carefully mixed using a glass rod.

The Erlenmeyer flasks are capped with carded cotton, covered with aluminum foil. They are sterilized in an autoclave at 121°C for 20 minutes.

After cooling, the sterilized Erlenmeyer flasks are inoculated with yeast suspension and incubated at 40°C for 112 hours.

1.7 Enzyme Extraction

After fermentation, a 25 ml of Tween 80 solution (0.02%) is added on a 2.5 g of the fermented substrate. The mixture is mixed for 5 minutes and the resulted solution is put in a conical tube then centrifuged at 10,000 rpm at 4°C for 10 minutes.

The supernatant is then recovered and filtered through a filter paper (Whatman No. 1), to remove the particles from the substrate.

This process is repeated until the fermented substrate is finished.

The filtrate obtained is a crude enzymatic extract that should be stored in the freezer until later use.

2. Analytical methods

2.1 Determination of dry matter

The dry matter of the waste is determined by drying a 5 g sample introduced into pre-dried weighed glass Petri dishes and placed in an oven at 50°C for 48 hours until constant weight.

DM (%): [(FW-DW) /FW] X 100

DM: Dry matter **FW**: Fresh weight **DW**: Dry weigh

2.2 Protein assay

2.2.1 Protein content determination (Lowry et al., 1951)

The principle of protein determination is based on the result of two reactions:

- The first is the Biuret reaction to increase detection sensitivity, where the presence of copper sulphate in an alkaline medium lead to the formation of a complex between the cupric ion and the peptide bond in the protein.

- The second is the reaction with the Folin-Ciocalteu reagent which reacts on the aromatic amino acid tyrosine and tryptophan present in proteins and is reduced to a complex with a blue-violet color proportional to the quantity of amino acids present in the medium and which can be detected between 650 and 750 nm.

After dosing, the protein level is calculated by reference to a calibration curve established from a standard Bovine Serum Albumin (BSA) solution at 500 µg/ml. Each assay is performed in duplicate **(Appendix 2).**

2.2.2 Dosage of proteolytic activity

After the hydrolysis of proteins by proteases they release amino acids and simple peptides, the non-hydrolyzed molecules are precipitated by TCA **(ANSON 1938)**. Tyrosine is

an aromatic amino acid found in all proteins; it is used as standard for colorimetric assay of protease activity using folin reagent. The latter reacts with tyrosine and tryptophan to give a blue color. **(The protocol is described in appendix 2).**

The activity is calculated by reference to a calibration curve established using tyrosine as a standard **(appendix2).**

One unit (U) of protease is the equivalent of 1 µg of tyrosine released over 1 h of time per 1 ml of an enzyme solution.

2.3 Partial purification of the enzyme

The partial purification of the crude enzyme extract involves a fractional precipitation of the proteins by ammonium sulfate $(NH_4)_2 SO_4$ then a dialysis with a semi-permeable.

2.3.1 Ammonium sulfate precipitation

It was found that the proteins present in a solution can be precipitate with a certain range of salt concentration depending on the nature of the protein to be extracted.

It is one of the fastest methods to recover proteins in concentrated form while retaining their biological activities (Walsh and Headon, 1994)

Operating mode

To determine the best ammonium sulfate concentration for the purification of protease three set of solutions were prepared at different saturation

At 60 % saturation (10.83 g $(NH_4)_2SO_4$ / 30 mL crude enzyme)

At 70 % saturation (13.08 g $(NH_4)_2SO_4$ / 30 mL crude enzyme)

and at 80 % saturation (15.48 g $(NH_4)_2SO_4$ / 30 mL crude enzyme)

Each set was stirred slowly overnight at 4 C° until homogeneous, afterwards each was centrifuged at 10000 rpm for 30 min.

After the centrifugation process is complete, the supernatant and pellets are separated and the enzyme activity level is measured using Anson method while the protein content is measured by Lowry method.

2.3.2 Dialysis

Principle

Dialysis is a separation method based on the movement of molecules from the more concentrated medium to the less concentrated medium through a semi-permeable membrane. Only molecules with dimensions smaller than the diameter of the membrane pores can diffuse

to both sides and reach equilibrium with the total volume of solution in the system, such as the volume of solvents, salts, and small metabolites. In contrast, macromolecules such as proteins are non-diffusible and they will remain in the same membrane compartment as at the start of the experiment (Rapalli and Singhvi, 2021; Soria et al., 2012).

Operating mode

The precipitated proteins suspended in a reduced volume of sodium citrate buffer (0.1 M; pH 5) are dialyzed against the same buffer, using a semi-permeable membrane under gentle stirring at 4°C for 24 h.

2.3.3 Lyoufilisation

Principe

Lyophilisation is a process commonly used in the food industry, biological samples preservation, and pharmaceutical applications, being a dehydrating technique suitable for heat-sensitive samples. This consists of the removal of water from a sample, either being from a solution or materials. During the process, the water is removed from previously frozen samples directly into vapor by sublimation, in decreased temperature and pressure conditions.

In this case this principle is used to rid the crude extract of any unnecessary liquids like the buffer with the goal of raising the protein concentration

Operation mode

The equipment used is Labconco FreeZone 1 liter Freeze Dryer

It was loaded with three batches of three Petri dishes each filled with 5mL of crude extract

The lyoufilisation time of each batch was 6 hours After which the remains on the dishes are collected and stored in an Eppendorf tube **(Figure 14).**

Figure 14: Labconco FreeZone 1 litre freeze dryer.

2.4 SDS PAGE Electrophoresis

The electrophoresis gel technique under denaturing conditions (Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis or SDS page) is used to determine the number of protein subunits and their molar mass(Laemmli, 1970).

The protein sample is treated with a reducing agent (β-mercaptoethanol) which breaks down the disulfide bounds and an anionic detergent (SDS) which denatures proteins and gives them an envelope of negative charges(Hames et al., 2000).

All protein fragments are separated according to their molecular weight, determined using markers.

This technique makes it possible to determine the molecular weight of the subunits forming a protein.

The samples are fractionated by 10% polyacrylamide gel electrophoresis.

The samples used were

The crude extract

The partially purified extract at 80%, 70%, 60% saturation

The partially purified extract by lyoufilisation

3. Applications using protease

3.1 Determination of milk-clotting activity

Milk-clotting activity was determined according to the methods described by (Arima et al., 2000)with slight modifications. The substrate 1 (10% milk in 0.01 M CaCl2) and the substrate 2 (milk) was prepared and the pH was adjusted to 6.5. The substrate was incubated for 5 min at 37°C.

3 tests were conducted:

test 1: tube1contain :2 ml substrate +1 0.2 ml Enzyme extract.
tube 2 contain :2 ml substrate 1.

test 2: tube1contain :6 ml substrate 1 + 0.6 ml Enzyme extract.
tube 2 contain :6 ml substrate 1 + 1.8 ml enzyme extract.
tube 3 contain: 6 ml substrate 1

test 3: tube1 contain :6 ml substrate 2 + 0.6 ml Enzyme extract.
tube 2 contain :6 ml substrate 2 + 1.8 ml enzyme extract.
tube 3 contain: 6 ml substrate 2.

the test tubes were incubated at 37°C, and being manually rotated from time to time. The milk clotting unit was defined as the amount of enzyme that coagulates 10 ml of the substrate in 40 minutes (Mohamed and Babiker, 2014).

MCA (U/ml) = (2400⁄ clotting time (sec)) × Dilution factor.

MCA: Milk Clotting Activity.

3.2 Detergent

The efficacy of the crude protease in stain removal was assessed using 4 pieces of white cloth (5 × 5 cm) stained separately with a proteinaceous mixture of eggs and chocolate and dried at 37 °C.

The stained clothes were placed in separate trays and the set-up was as follows:

Set 1 (control): 100 ml distilled water + the stained cloth.

Set 2: 100 ml distilled water + 1 ml ISIS detergent (7 mg/ml) + the stained cloth.

Set 3: 100 ml distilled water + 2 ml crude extract + the stained cloth.

Set 4: 100 ml distilled water + 1ml ISIS detergent + 2 ml crude extract+ the stained cloth.

All experimental sets were incubated at 45 °C for 30 min.

After the incubation, the clothes were rinsed with water without rubbing.

The stain removal activity was visually examined and compared after the cloth was dried. **(Figure 15).**

Figure 15: Cloth after adding the mixture.

3.3 Recovery of silver from waste X-ray photographic film

Two pieces of used X-ray films (2 x 2 cm) were washed with distilled water and wiped with cotton impregnated with ethanol then dried in an oven at 40 °C for 30 min

One film was submerged in 50 ml of crude extract and put in the oven at 40 °C

The other film was put under the same condition and used for a negative control, with 50 ml distilled water instead of enzyme solution.

The film was checked for decomposition of gelatinous coating after different incubation periods **(Figure 16).**

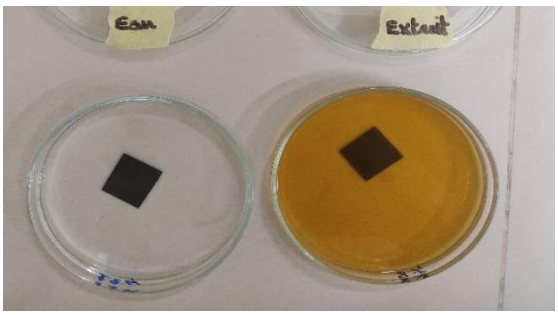

Figure 16: Used x-ray films submerged in crude extract and distilled water.

3.4 Action of protease on gluten

Principle of gluten dosage

The dosage of gluten is based on its solubility in salt water and on its agglomeration during mixing under a stream of water (Marchetti, 2012).

Operating mode

3 experiments were carried out to extract gluten: flour, wheat flour and wheat grains

- Weigh 25g of flour, then pour it into a container to which you add about 12 to 13 mL of distilled water. On the other hand, weigh 25 g of flour and add about 12 to 13 mL of enzymatic extract.
- Weigh out 25 grams of wheat flour, and pour it into a bowl to which you add about 12 to 13 ml of distilled water. On the other hand, weigh 25g of wheat flour and add about 12 to 13ml of enzymatic extract.
- Weigh 25 grams of wheat grains, mix in 50 ml of enzymatic extract, and then place in an incubator set at 60 degrees for 24 hours. After drying out, the mixture is crushed to make flour, which is then combined with 12 to 13 ml of distilled water. On the other hand, measure out 25 grams of wheat flour and add it to a bowl with 12 to 13 ml of distilled water.

after two to three minutes of kneading, and 15 minutes of rest, this dough is kneaded in the hand to transform it into a homogeneous dough that stretches perfectly. This dough is kneaded under a stream of water by compressing it slightly or by placing it above a sieve intended to retain the fragments of gluten that are entrained and recover them (the starch is therefore eliminated while the gluten gradually fuses to itself). When the gluten forms a

homogeneous mass, the water flow is increased to carry out the washing until transparent washing water is obtained. The gluten is then wrung out by compressing it strongly several times between the palms of the hands. Finally, the wet gluten is quickly weighed.
Dry gluten is obtained by drying wet gluten at a temperature of102°C, until a constant weight is obtained after approximately18hours.
We do the same process for wheat flour and wheat grains.

Expression of results

Wet gluten (GW) is expressed in grams per 100g of flour by:

$$GW=100(Mw/25)$$

Dry gluten (GD) is expressed in grams per 100g of flour by:

$$GD=100(Md/25)$$

Results and discussion

4. Strain characteristics

Macroscopic characteristics

After incubation for 48 hours at 30°C on YPGA solid media, the results are shown in **(Figure17)**, it appears that the P3 strain has colonies of a round form, smooth surface, creamy, shiny and white in color.

Figure 17: Cultivation of the strain *Clavispora lusitaniae* .

Microscopic characteristics

Their size ranges from 4 to 10 µm, many cells have buds attached to them indicating asexual reproduction through budding **(Figure 18).**

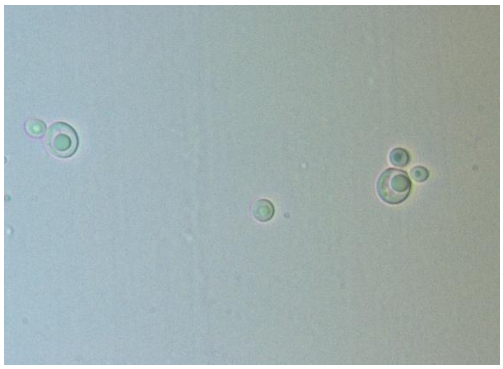

Figure 18: *Clavispora lusitaniae* under a microscope.

5. Protease production

The production of the protease from the strain *Clavispora lusitaniae* was carried out in SSF on a medium consisting of a mixture of bread and tomatoes leftovers, has been successful with the crude extract containing 75.6 ug/ml with enzymatic activity of 15960.6 UI/ml.

5.1 Extract partial purification

Ammonium sulfate and dialysis

The crude enzymatic extract from *Clavispora lusitaniae* characterized by proteolytic activity of 15960.6UI/ml was subjected to ammonium sulfate precipitation at different saturation (60%, 70%, 80%) then dialysis, in each stage of this partial purification protein concentration and enzymatic activity was measured, all results found have been summed up in the following **(table6)**.

Table 6: Purification Table.

stage	volume	Enzyme activity	Protein concentration	Total activity	Total protein	Specific activity	Purity	Yield
Crude extract	30	15960.6	75.6	478818	2268	211.12	1	100
Ammonium sulfate precipitation								
60%	4	411.76	18.66	520.20	74.64	22.066	0.104	0.108
70%	4.5	1735.2	17.27	7808.4	77.715	100.47	0.456	1.63
80%	4.25	12600	42.6	53550	181.05	295.77	1.40	11.18
Dialysis								
60%	2	209	12.5	4.18	25	16.72	0.76	0.09
70%	2.5	535.2	11.82	1338	29.55	45.28	0.45	0.28
80%	2.6	11400	25.2	29640	65.52	452.4	1.53	6.19

According to what was found the best saturation rate for ammonium sulfate precipitation of protease is 80% as it gives the highest proteolytic activity of 29640UI/ml and 6.19% yield, this value is slightly smaller than that studied for some protease from other yeast , such as *Pichia anomala CO-1* 7.2 % yield (Kim, 2019c).

5.2 SDS-PAGE

SDS-PAGE analysis of the samples from the extract resulted in the appearances of multiple bands indicating the presence of multiple enzymes with a molecular weight that ranges between 10 kDa to 28 kDa **(Figure 19)** ,this value is smaller than that studied for someprotease from other yeast ,*Pichia anomala CO-1* 30 KDa (Kim, 2019c),*Aureobasidium pullulans* 32 KDa (Ma et al., 2007b).

Given how far the extract migrated it can be concluded that the time given for the procedure should be shortened.

The presence of protease can be further investigated by relying on its specific enzymatic activity using zymography technique.

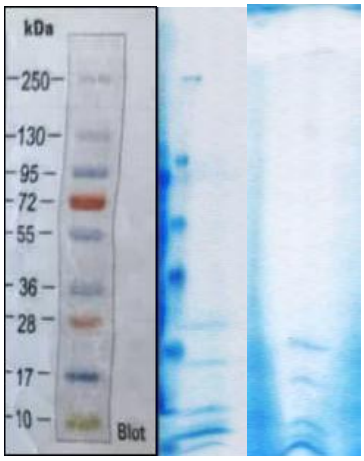

Figure 19: SDS-PAGE analysis.

6. Applications

6.1 Determination of milk-clotting activity:

After 4,8, 24 hours of incubation our enzyme showed no apparent coagulant activity.

It may be that the protease enzyme produced by *Clavispora lusitaniae* has no milk clotting activity or it is very small it doesn't make a difference

The conditions of the experiment are not favorable as milk clotting activity can be affected by many factors, the foremost being pH and temperature. A study of the effects of pH and temperature on the milk-clotting activity and proteolytic activity of the proteases showed that they exhibited good pH stability from pH 5.5 to 7.5 and good thermal stability at temperatures from 50 to 70°C (Luo et al., 2018).

The incubation time is not sufficient

milk clotting activity could be due to differences in the composition of substrates, mineral supplements, incubation time, and/or moisture content used in SSF (Mamo et al., 2020).

With this, we can assume that the protease produced by *Clavispora lusitaniae* is not suitable for cheese industry but further research is required to prove this.

6.2 Results of silver

After two hours, small black particles were observed floating in the crude extract while the sides of the film turned more transparent and after 24 h, the gelatin layer was completely removed leaving the polyester film clear **(Figure 20).**

From the results obtained in this attempt, it can be deduced that the protease from *Clavispora lusitaniae* was effective in breaking down the gelatin making it potentially a good alternative to harmful chemicals in the process of recovering silver from used X-ray films.

The study of Pathak and Deshmukh (Pathak and Deshmukh, 2012) showcased similar results for it took them 24h for the gelatin layer to break and for the silver to be extracted while Foda took 1h to do so (Foda and Ali, 2013), some even managed to finish the treatment for as short as 3 min like Seid or 15 min like Choudhary (Choudhary and Vishwavidylaya, 2013; Seid, 2011)

Figure 20: X-ray film put in the ezyme extract after 24 h.

6.3 Detergent results

One of the most important commercial applications of protease is its use in the detergent industry; hence, the stain removal capacity of protease is assessed visually using eggs and chocolate-stained fabrics.

(Figure 21) shows that the presence of crude enzyme with the commercial detergent ISIS showed good stain removal capacity; this suggests the combination (enzyme -detergent) seems to enhance the performance of the detergent, which can make it a great supplement ingredient to be put alongside regular chemical detergents.

These results go in hand with other studies on different types of proteases as the test conducted of their stability and compatibility with various commercial detergents support their usage as additives, this was the case with proteases from *Aspergillus terreus*

(Ali U F,2008) and *Bacillus circulans* (Jaswal, R and Kocher, G, 2006)and protease from *B. licheniformis* by Jyothi Bezawada, (Bezawada, 2010).

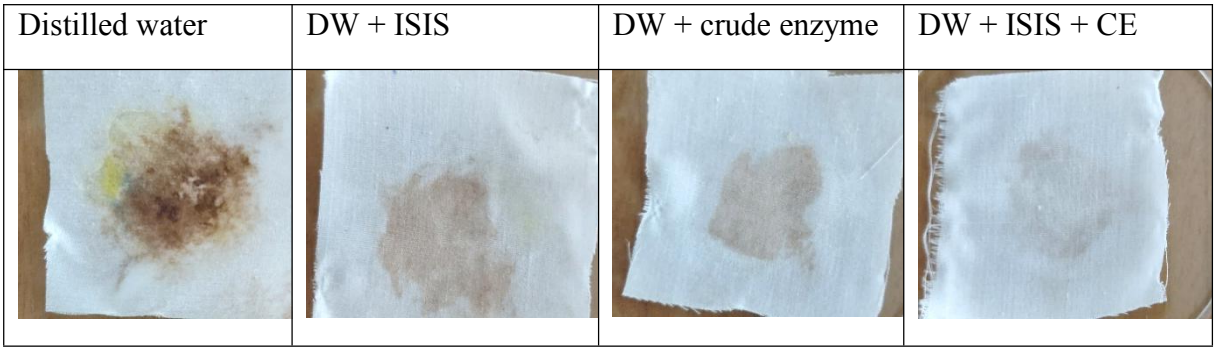

Figure 21: Results of the detergent application.

6.4 Action of protease on gluten

The results of the experiment show that overall, the weight of gluten is less in the dough that has been treated with the enzyme extract compared to the weight of gluten in the dough that has been kneaded with distilled water.

The enzyme extract degrade respectively 0.28 %, 1%, 2.01% of gluten of flour (*Triticum aestivum*),wheat flour (*Triticum durum*),wheat grains (*Triticum durum*) **(Figure 22) (Figure 23) (Figure 24).**

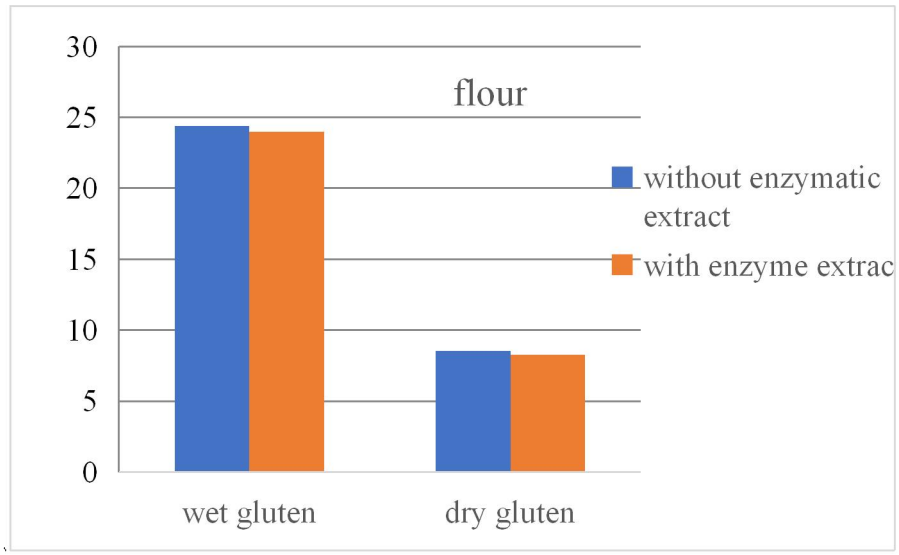

Figure 22: Percentage of gluten in flour after and before treatment by extract enzyme.

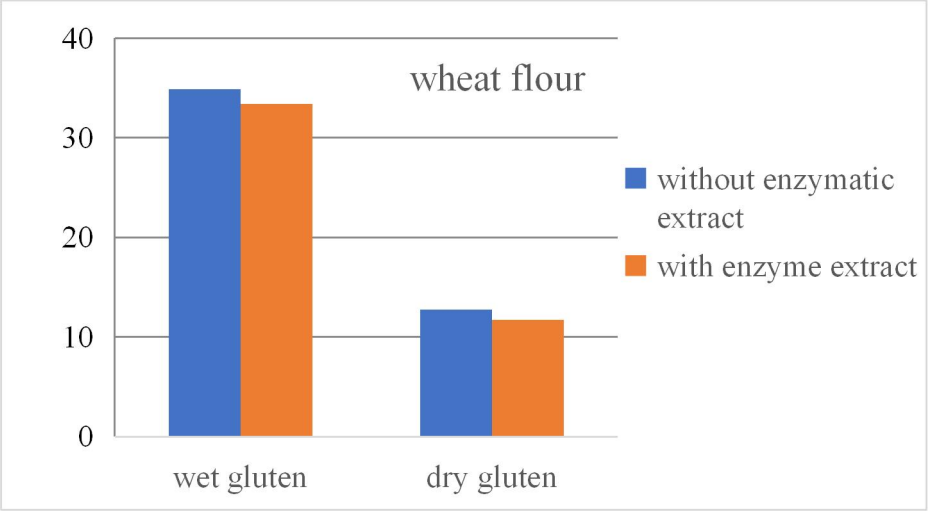

Figure 23: Percentage of gluten in wheat flour after and before treatment by extract enzyme.

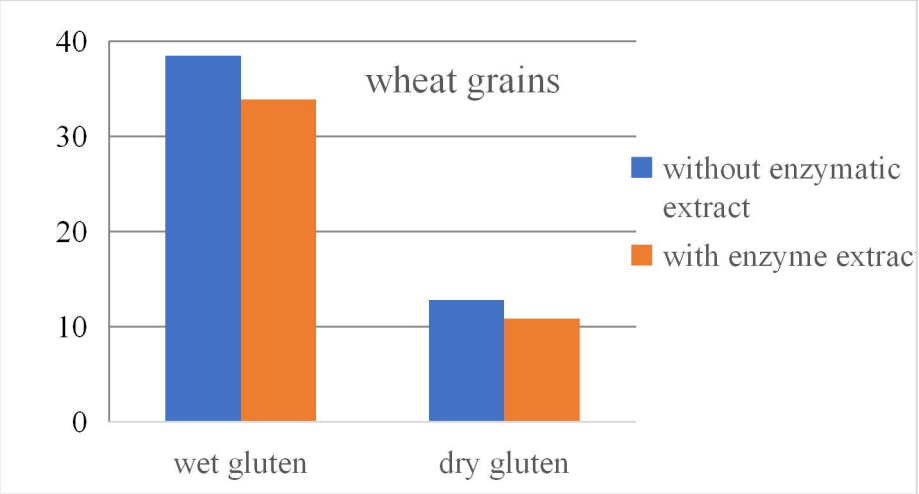

Figure 24: Percentage of gluten in wheat grains after and before treatment by extract enzyme.

The proteolytic activity of strain *Clavispora lusitaniae* presented an interesting capacity to degrade gluten.

General conclusion

The objective of our study to produce protease of the strain *Clavispora lusitaniae* in optimal conditions, and test the properties of crude proteases in the field of industry and biotechnology.

In the first part, the production of protease by the yeast *Clavispora lusitaniae* by solid state fermentation (SSF) and the use of tomato waste and leftover bread as substrates were studied under optimal conditions of 0.5×10^6 of inoculum, 96.82% humidity, 112 hours of incubation, 40 °C temperature, and pH 5.

After fermentation and extraction of the enzyme, the proteolytic activity was measured15960.6 UI/ml and the concentration is 75.6ug/ml.

After partial purification of the protease by several techniques: Precipitation by ammonium sulphate, Dialysis, electrophoresis and lyophilization.

Ammonium sulphate precipitation indicates that the majority of the proteins were precipitated in 80% as it gives the highest proteolytic activity of 29640UI and 6.19% yield.

Electrophoresis indicates an appearance of multiple bands indicating the presence of multiple proteins with a molecular weight that ranges between 10 kDa to 28 kDa.

In the second part, four important applications were discussed, to understand the uses of protease.

The protease enzyme showed an interesting effect on gluten, which has been shown to degrade respectively 0.28 %, 1%, and 2.01% of gluten in flour (*Triticum aestivum*), wheat flour (*Triticum durum*), wheat grains (*Triticum durum*). It also played a good role in the field of silver recovery, and the possibility of using it in the field of commercial detergent industry. For the experiment of milk clotting activity our enzyme showed no coagulant activity so we can't use it in cheese industry.

Therefore, we conclude that our strain produces proteases with a good property (proteolytic activity), which is important in different fields of industry and biotechnology.

The objectives set in this work are achieved, but they have opened a path of research towards other perspectives such as:

-Using zymography technique.

-Total purification.

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Appendices

Appendix 1

Yeast Extract Peptone Glucose Agar (YPGA)

2% agar medium dissolve in 1 liter of distilled water:

20 g glucose.

10 g of peptone.

5 g of yeast extract.

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

Preparing the buffer solution

Sodium Citrate Buffer (pH)

- Solution A: citric acid $C_6H_8O_7$ (0,1M).
- Solution B: the disodium Na_2HPO_4 (0,2M).

The pH is determined by the pH meter.

Appendix 2

Protein assay

Solutions preparation

- Solution A: 2% of Na_2 CO3 in 0.1 N NaOH (0.4% in distilled water)
- Solution B: 2% tartrate in distilled water
- Solution C: 1% CuSO4 (5H2O) in distilled water
- Solution D: 0.5 mL C + 0.5 mL B + 50 mL A (must be prepared right before utilization)
- Folin-Ciocalteu diluted three times (one volume of folin with two volumes of distilled water)
- BSA stock solution of 500 µg/ml for the standard curve

The assays protocol **(Table 7):**

Table 7: Protein assay protocol.

0.5 ml enzymatic extract (diluted 1:10 in distilled water) 2.5 ml of solution D
Agitation and Incubation in an ambient temperature for 10 minutes
0.25 ml of folin-ciocalteu diluted (1:3) at 50%
Agitation and Incubation in an ambient temperature in the dark for 30 minutes
Read the absorbance at 650 nm

Standard curve preparation (BSA)

The standard range is established from a BSA stock solution with a concentration between 0 and 500 µg/ml. The reaction mixture of different concentrations is prepared according to the same protocol described above and as indicated in the following **(Table 8):**

Table 8: Standard curve preparation protocol.

Tube	**Blanc**	**1**	**2**	**3**	**4**	**5**
BSA (ml)	0	0,2	0 ,4	0,6	0,8	1
Distilled water (ml)	1	0,8	0,6	0,4	0,2	0
Solution D (ml)	5	5	5	5	5	5
Agitation and Incubation in an ambient temperature for 10 minutes						
Folin (ml)	0,5	0,5	0,5	0,5	0,5	0,5
Agitation and Incubation in an ambient temperature for 30 minutes						
Read absorbance at 650 nm						

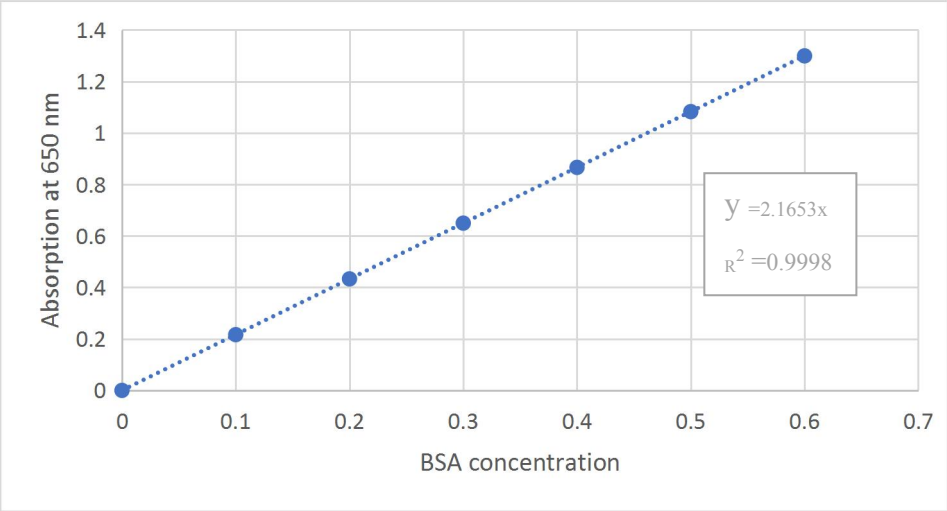

Figure 25: BSA standard curve.

Dosageof Proteolytic activity:

❖ **Necessary solutions:**

- ✧ 2.5% casein solution dissolved in 0.02M sodium citrate.
- ✧ Citrate / sodium buffer, (0.1M / 0.2 M), pH =5.
- ✧ 4% TCA solution.
- ✧ 2% Na_2 CO_3 solution in 0.1N NaOH.
- ✧ Folin-Ciocalteu's reagent diluted to 50%.
- ✧ Tyrosine stock solution with a concentration of 100µg/ml (0.01%) dissolved in TCA 4% trichloroacetic acid solution for calibration.

The dosage of protein activity: is carried out in two phases:

Phase 01: The Enzymatic Reaction: Preparation of the reaction mixture.**(Table 9):**

Table 9: Protocol of phase 1 of enzyme activity assay.

• 0.375 ml of citrate / sodium buffer (0.1 M / 0.2 M), pH = 5. • 0.25ml of the enzymatic extract thawed just before the assay and diluted (1/10th). • o 0.625ml 2.5% casein substrate dissolved in sodium citrate buffer (0.02M).
Shaking and Incubation in the water bath at 40C° for 30 minutes.
• o 2.5 ml 4% TCA, to stop the reaction.
Shaking and standing for 10 minutes to allow complete precipitation of unhydrolyzed casein.
Filter on Whatman paper

NB: The blanks are prepared in the same way as the sample except that here the TCA is added before the substrate and without incubation in the water bath.

Phase 02: Activity colorimetric dosage: After filtration, the non-protein nitrogen compounds that are in the soluble phase are dosed. **(Table 10):**

Table 10: Protocol of phase 2 of enzyme activity assay.

2,5 ml of Na2CO3 2% in NaOH 0.1N. 0.5 ml of filter. 0.25 ml Folin diluted 1/4 in distilled water.
Shake and incubate at room temperature, in the dark for 30 minutes.
Absorbance reading at 750 nm.

Preparation of the curve:

The standard range is established from a tyrosine solution, concentrations are

between 0 and 100 µg/ml according to the following **(Table 11):**

Table 11: Protocol of the preparation for TCA standard curve.

Concentrations of dilutions (µg/ml)	0	20	40	60	80	100
Solution of Tyrosine (ml)	0	0.1	0.2	0.3	0.4	0.5
TCA (ml)	0.5	0.4	0.3	0.2	0.1	0
Na_2CO_3(ml)	2.5	2.5	2.5	2.5	2.5	2.5
Shake and incubate for 10 minutes at room temperature.						
Diluted reactive ½ (ml)	0.25	0.25	0.25	0.25	0.25	0.25
Shake and incubate at room temperature for 30 minutes.						
Absorbance reading at 750 nm.						

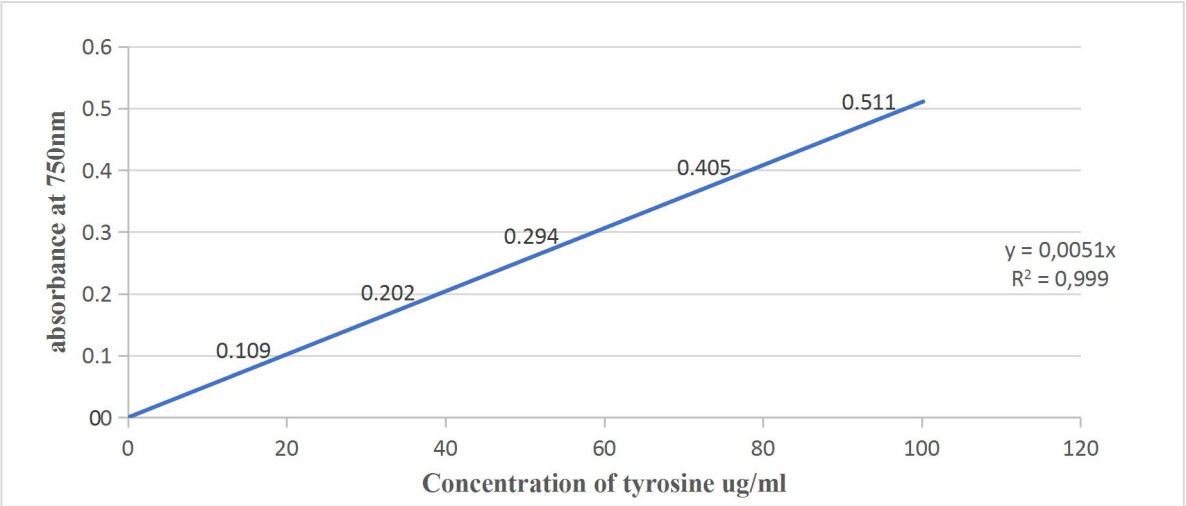

Figure 26: TCA standard curve.

Summary

ملخص

الهدف من دراستنا هو إنتاج إنزيم البروتياز محليًا من سلالة محلية *Clavispora lusitaniae* عند الظروف المثلى باستخدام ركائز غير مكلفة، واختبار خصائص البروتياز الخام في مجال الصناعة والتكنولوجيا الحيوية.

تم إنتاج البروتيز *Clavispora lusitaniae* CBS 6936 T عن طريق التخمير الصلب على خليط من 50 ٪ من بقايا الخبز و50 ٪ من نفايات الطماطم. أعطى الإنتاج الأمثل نشاط تحلل للبروتين قدره 15960.6 وحدة دولية / مل وتركيز 75.6 ميكروجرام / مل.

يشير ترسيب كبريتات الأمونيوم إلى أن أفضل تنقية وأفضل مردود (1.40 و 11.18 على التوالي) تم الحصول عند ترسيب 80٪.

تشير نتائج الهجرة الكهربائية إلى ظهور عدة شرائط تشير إلى وجود عدة بروتينات بوزن جزيئي يتراوح بين 10 كيلو دالتون و 28 كيلو دالتون.

تم إثبات فعالية البروتياز في التجارب التالية:

إعادة تدوير الفضة من ورق التصوير، حيث قام بفصل طبقتين من الجيلاتين. تأثيره على الغلوتين يجعله مناسبًا لإنتاج قمح منخفض الغلوتين لعلاج مرضى الاضطرابات الهضمية. تم توضيح أهميته كعنصر جيد في تصنيع المنظفات التجارية.

الكلمات المفتاحية: الإنتاج،البروتياز،الخميرة، الرحلان الكهربائي، ترسيب كبريتات الأمونيوم، التطبيق

Clavispora lusitaniae CBS 6936 T

Résumé :

L'objectif de notre étude est de produire localement l'enzyme protéase à partir d'une souche locale *Clavispora lusitaniae* dans des conditions optimales en utilisant des substrats peu coûteux, et de tester les propriétés des protéases brutes dans le domaine de l'industrie et des biotechnologies.

La production de la protéase de *Clavispora lusitaniae CBS 6936 T a été réalisée en* fermentation solide sur un mélange de 50% restes de pain et 50% déchets de tomate. La production optimisée a donné une activité protéolytique de 15960,6 UI/ml et une concentration de 75,6 µg/ml.

La précipitation au sulfate d'ammonium indique que le meilleur degré de purification et le meilleur rendement (1.40 et 11,18, respectivement) ont été obtenus à 80%.

L'électrophorèse indique l'apparition de plusieurs bandes indiquant la présence de plusieurs enzymes avec un poids moléculaire compris entre 10 kDa et 28 kDa.

L'efficacité de la protéase a été prouvée dans les expériences suivantes :

Le recyclage de l'argent du papier photographique, où il a séparé les deux couches de gélatine. Son effet sur le gluten, le rend approprié pour produire du blé à faible teneur en gluten pour le traitement des patients atteints de troubles cœliaques. Son importance en tant que bon ingrédient dans la fabrication de lessive commerciale a été montrée.

Mots clés : production, protéase, levure, *Clavispora lusitaniae CBS 6936 T*, électrophorèse, précipitation au sulfate d'ammonium, application.

University year : 2022-2023	Presented by :Allem Khadidja. Mosbah Saba.

The production of protease from the yeast *Clavispora lusitaniae* and some of it appliations

Memoire to obtain Master degree in Biochemistry

The aim of our study is to locally produce the protease enzyme from a local *Clavispora lusitaniae* strain under optimal conditions using inexpensive substrates and to test the properties of crude proteases in the field of industry and biotechnology.

The production of *Clavispora lusitaniae* CBS 6936 T protease was carried out in solid fermentation on a mixture of 50% bread scraps and 50% tomato waste. Optimized production gave a proteolytic activity of 15960.6 IU/ml and a concentration of 75.6 ug/ml.

Ammonium sulfate precipitation indicated that the best degree of purification and yield (1.40 and 11.18, respectively) were obtained at 80%.

Electrophoresis showed the appearance of several bands indicating the presence of several enzymes with molecular weights ranging from 10 kDa to 28 kDa.

Protease efficiency was demonstrated in the following experiments:

Recycling silver from photographic paper, where it separated the two gelatin layers. Its effect on gluten makes it suitable for producing low-gluten wheat for the treatment of celiac patients. Its importance as a good ingredient in the manufacture of commercial laundry detergents has also been demonstrated.

Key words: production, protease, yeast, *Clavispora lusitaniae strain CBS 6936 T*, Electrophoresis, Ammonium sulphate precipitation, application.

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