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α-Amylase Study in *Geotrichum candidum*: Optimization of production, Purification and Enzyme Characterization

PhD Thesis

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Field: Biological Sciences
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Dedication

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Truth is more valuable if it takes you a few years to find it

(Jules Renard)

Abstract: Enzymes, as biocatalysts, are highly valued for their industrial applications. Among the various sources available, microbial enzymes, and in particular those from yeast, are of great interest due to their polyvalence and numerous biotechnological applications. This study aims to investigate the production of enzymes with high biotechnological potential from a local yeast strain. After isolation from various samples collected in different regions, 100 strains were grown and evaluated for their enzymatic capacity on substrates such as soluble starch, carboxymethylcellulose, Tweens and olive oil, using rapid plate detection methods at 28°C on YPGA and YMA media. Yeast PO27, selected for its significant α-amylase production as well as its lipolytic and esterolytic activities, and was identified as *Geotrichum candidum* by analysis of the D1/D2 region of the 26S ribosomal RNA sequence. To address the economic challenge associated with amylase production, this study focused on the use of inexpensive substrates such as olive pomace, potato peelings, leftover bread and mastic waste for solid-state and submerged fermentation. The results showed that olive pomace was the best substrate, achieving a maximum α-amylase production of 180.71 U/g through solid-state fermentation. Optimizing culture conditions with a central composite design, production was increased to 412.94 U/g under specific conditions of humidity, malt extract and $CaCl_2$. Physicochemical characterization of purified α-amylase revealed optimal activity at pH 5 and 70°C, with remarkable stability in the presence of Mg^{2+} and Tween 80, and resistance to surfactants and organic solvents. The enzyme showed notable efficacy in cotton desizing at room temperature, demonstrating its potential for low-cost, efficient industrial applications.

Keywords: α-Amylase, *Geotrichum candidum* PO27, Olive pomace, Statistical optimization, Purification and characterization

Résumé: Les enzymes, en tant que biocatalyseurs, sont largement prisées pour leurs applications industrielles. Parmi les différentes sources disponibles, les enzymes microbiennes, et notamment celles des levures, suscitent un vif intérêt en raison de leur polyvalence et de leurs nombreuses applications biotechnologiques. Cette étude vise à explorer la production d'enzymes à fort potentiel biotechnologique à partir d'une souche de levure locale. Après isolation à partir de divers échantillons collectés dans différentes régions, 100 souches ont été cultivées et évaluées pour leur capacité enzymatique sur des substrats tels que l'amidon soluble, la carboxyméthylcellulose, les Tweens et l'huile d'olive, utilisant des méthodes de détection rapide sur plaque à 28°C sur milieux YPGA et YMA. La levure PO27, sélectionnée pour sa production significative d'α-amylase ainsi que ses activités lipolytique et estérolytique, a été identifiée comme *Geotrichum candidum* par l'analyse de la région D1/D2 de la séquence de l'ARN ribosomique 26S. Pour surmonter le défi économique associé à la production d'amylases, cette étude s'est concentrée sur l'utilisation de substrats peu coûteux tels que les grignons d'olive, les pelures de pommes de terre, les restes de pain et les déchets de lentisque pour la fermentation en milieu solide et submergée. Les résultats ont montré que les grignons d'olive étaient le meilleur substrat, permettant d'atteindre une production maximale d'α-amylase de 180.71 U/g par fermentation en milieu solide. En optimisant les conditions de culture avec un plan composite central, la production a été augmentée à 412.94 U/g sous des conditions spécifiques d'humidité, d'extrait de malt et de $CaCl_2$. La caractérisation physicochimique de l'α-amylase purifiée a révélé une activité optimale à pH 5 et à 70°C, avec une stabilité remarquable en présence de Mg^{2+} et Tween 80, et une résistance aux surfactants et solvants organiques. L'enzyme a montré une efficacité notable dans le désencollage du coton à température ambiante, démontrant ainsi son potentiel pour des applications industrielles à faible coût et efficaces.

Mots clés : α-Amylase, *Geotrichum candidum* PO27, Grignons d'olive, Optimisation statistique, Purification et caractérisation.

ملخّص: تحظى الإنزيمات باعتبارها محفزات حيوية بتقدير واسع النطاق لتطبيقاتها الصناعية. من بين المصادر المتعددة المتاحة، تحظى الإنزيمات الميكروبية، وخاصة إنزيمات الخميرة، باهتمام كبير نظرًا لتعدد استخداماتها وتطبيقاتها التكنولوجية الحيوية المختلفة. تهدف هذه الدراسة إلى دراسة إنتاج إنزيمات ذات إمكانات عالية في مجال التكنولوجيا الحيوية من سلالة خميرة محلية. بعد عزل عينات مختلفة جُمعت من مناطق متنوعة، تمت زراعة 100 سلالة وتقييم قدرتها الإنزيمية على ركائز مثل النشا القابل للذوبان وكربوكسي ميثيل السليلوز والتوينز وزيت الزيتون، وذلك باستخدام طرق الكشف السريع على ألواح الطبق عند درجة حرارة 28 درجة مئوية على وسائط YPGA وYMA. تم التعرف على الخميرة PO27، التي تم اختيارها لإنتاجها الكبير من إنزيم ألفا-أميلاز بالإضافة إلى أنشطتها المحللة للدهون والأسترة على أنها *Geotrichum candidum* من خلال تحليل منطقة D1/D2 من تسلسل الحمض النووي الريبوسومي S26. وللتغلب على التحديات الاقتصادية المرتبطة بإنتاج إنزيم الأميلاز، ركزت هذه الدراسة على استخدام ركائز منخفضة التكلفة مثل ثفل الزيتون وقشر البطاطا وبقايا الخبز ومخلفات اللنتيسك (الضرو) للتخمير في الحالة الصلبة والتخمير المغمور. أظهرت النتائج أن ثفل الزيتون كان أفضل ركيزة حيث بلغ الحد الأقصى لإنتاج إنزيم ألفا-أميلاز 180.71 وحدة/غم بالتخمير في الحالة الصلبة. ومن تحسين ظروف الاستزراع باستخدام مستوى مركب مركزي مركب، تمت زيادة الإنتاج إلى 412.94 وحدة/غرام في ظل ظروف محددة من الرطوبة ومستخلص الشعير وكلوريد الكالسيوم. كشف التوصيف الفيزيائي والكيميائي لإنزيم ألفا-أميلاز المنقى عن نشاط مثالي عند درجة الحموضة 5 ودرجة الحرارة 70 °م، مع ثبات ملحوظ في وجود Mg^{2+} وTween 80، ومقاومة للمواد الخافضة للتوتر السطحي والمذيبات العضوية. كما أظهر الإنزيم فعالية ملحوظة في نزع محتوى القطن في درجة حرارة الغرفة، مما يدل على إمكاناته في التطبيقات الصناعية الفعالة ومنخفضة التكلفة.

الكلمات المفتاحية :ألفا أميلاز،PO27 *Geotrichum candidum* ، ثفل الزيتون، التحسين الإحصائي، التنقية والتوصيف.

Contents table

Section 3:Results and discussion

Abbreviation list

AEC: Anion Exchange Chromatography

AI: Amylolytic Index

ASP: Ammonium Sulfate Precipitation

CMC: Carboxymethyl Cellulose

DNSA: Dinitro Salicylic Acid

EDTA: Ethylenediamine Tetraacetic Acid

EtBr: Ethidium Bromide

GFC: Gel Filtration Chromatography

LPMOs: Lytic Polysaccharide Monooxygenases

MC: Mastic Oil Cake

NCBI: National Center for Biotechnology Information

OFAT: One Factor At Time

OP: Olive Pomace

OS: Olive Forest Soil

OSP: Organic Solvent Precipitation

PBD: Plackett and Burman Design

RSM: Response Surface Methodology

RW: Olive Rinse Water

SDA: Sabouraud Dextrose Agar

SDS: Sodium Dodecyl Sulfate

Smf: Submerged Fermentation

SSF: Solid-State Fermentation

TPA: Tween Peptone Agar

TPP: Three-Phase Partitioning

TW: Thermal Water

VS: Vegetable Smen

YGA: Yeast Extract Glucose Agar

YM: Yeast Extract Malt Extract

YPCA: Yeast Extract Peptone Carboxymethyl Cellulose Agar

YPDA: Yeast Extract Peptone Dextrose Agar

YPSA: Yeast Extract Peptone Soluble Starch Agar

Illustration list

Tables list

Introduction

Introduction

The global demand for industrial enzymes has been consistently increasing, with a market value of approximately $0.31 billion in 1960, rising to $4.9 billion in 2015, $6 billion in 2020, and anticipated to exceed $9 billion USD by 2027. This growth has translated into substantial success for numerous enzyme companies (Pellis *et al*., 2018 ;Costa-Silva et *al*., 2021 and Okpara, 2022). Enzymes are produced by plants, animals, and microorganisms, however microbial enzymes gaining prominence due to their accessibility, rapid growth, active and stable nature, high yield on cost-effective media, shorter production time, and ease of production through recombinant DNA technology using microbes as host cells (Chávez-Camarillo *et al*., 2022; Raina *et al*., 2022 and Vachher *et al*., 2021) . Microbial amylases catalysis the hydrolysis of internal α-1, 4-glycosidic linkages in starch into reducing sugars. These enzymes are an important enzyme group used in many starch-based industries, most specifically in fermentation, animal feed, brewing, detergents, textiles, food, baking, distillation and the paper industry. α-Amylase are more popular owing to advantages such as their numerous cleavage sites, faster than β-amylase (Erfanimoghadam and Homaei, 2023 and Reddy *et al*., 2003).

Most of microorganisms are unable to produce enzymes stables under harsh condition. α-amylase from thermophilic microorganisms are more resistant compared to enzymes from mesophilic microorganisms, to their stability at a wide pH range, high temperatures and in various detergents and surfactants (Sharif *et al.*, 2023). Indeed, amylases from yeast known for their confirmed thermoresistance, which qualifies it for predominant use in industries, in recent years, yeasts have been discovered to be widespread in nature and have emerged as a significant source of enzymes, including α-amylase, lipase, cellulase, and esterase. Additionally, they exhibit characteristics such as high lipid production capacity, short fermentation cycles, independence from climatic conditions, tolerance to low pH values, with their ability to grow on a diverse range of substrates (Vyas and Chhabra, 2017).

The production of enzymes, including α-amylase, using synthetic media is a costly and economically inefficient process. To reduce the enzyme cost, there is a need for low-cost alternatives to meet industrial demands. Researchers have explored the use of agricultural by-

products like potato peels, bread waste, and oil cakes as substitute substrates, aiming to replace the expensive media traditionally used in both submerged fermentation (SmF) and solid-state fermentation (SSF) processes (Ramachandran *et al*., 2004; Benabda *et al*., 2019 and Olakusehin and Oyedeji, 2022) .

The industrial-scale production of α-amylase from *G. candidum* has not been extensively explored. Although the extracellular amylase of *G. candidum* was reported more than decade ago (Attanayaka *et al*., 2009) subsequent studies in this area have been limited. Therefore, to address these scientific gaps, this study aims to isolate yeasts with notable enzymatic potential for biotechnological applications. It also aims to produce cost-effective α-amylase using locally available and environmentally friendly substrates. Additionally, the optimization of fermentation conditions will be explored to understand their impact on strain enzymatic production, with the goal of enhancing both yield and product quality. The "one factor per time", Plackett-Burman design and response surface methodology with central composite design of Box-Wilson optimisation approaches were used to achieve maximum enzyme production under solid-state fermentation conditions. Furthermore, kinetics study on amylase production was carried out in order to select best time conditions. According to our research, this is the first time that α-amylase production in *Geotrichum candidum* PO27 has been optimised using very well selected parameters and still with statistical designs. Furthermore, characterization of thermostable purified *Geotrichum candidum* PO27 α- amylase newly isolated from olive pomace was also evaluated in laboratory conditions.

This PhD thesis is structured into two main sections: the first provides an overview of current knowledge on *Geotrichum candidum* yeasts and alpha-amylase enzymes, while the second focuses on experimental techniques, methodologies, and the presentation and discussion of results. In this part, the study will investigate the development of a novel yeast enzyme for industrial biotechnology use by initially isolating and identifying amylolytic yeasts from eastern Algeria. Subsequently, the most effective yeast strain for α-amylase production will be selected. Research in this section focuses on enzyme production, optimization, and kinetic studies, alongside efforts in enzyme purification, exploring biochemical characteristics, and assessing its response to different metal ions and chemical reagents. The thesis aims to comprehensively evaluate the enzyme's industrial application and validate its efficacy.

Literature review

1. General overview of yeasts

Yeasts can be defined as single-celled eukaryotes. They are classified in the Fungi family, which also contains multicellular organisms such as molds and fungi. These organisms belong to the two major groups of higher fungi: Ascomycota and Basidiomycota. However, there are several "yeast-like" forms that can have both filamentous and unicellular phases in their life cycle (Deák and Péter, 2013). Yeasts, whether ascomycetes or basidiomycetes, are generally known for their asexual reproduction by budding primarily or by fission occasionally, and for their sexual characteristics not included in the fruiting bodies (Kurtzman *et al*., 2011). Yeasts' reproductive processes contribute to their adaptability and survivability in a variety of environments. Yeasts are less widely distributed than bacteria in the natural environment. In general, they may have been isolated from soil, water, plants, animals, and insects(Walker, 2009). Biotech yeasts offer a several advantages to their users because of their easier cultivation, higher specific growth rate and shorter shelf-life (Chávez-Camarillo *et al*., 2022).

They are important in a variety of fields, particularly biology, and biomedical research, in addition to their traditional roles in food and fermentation industries. As a result, modern yeast biotechnology provides products for many commercially important areas applications including food, beverages, chemicals and enzyme manufacturing, pharmaceuticals, agriculture, and the environment (Walker, 2009).

2. *Geotrichum candidum* yeast

2.1. Background

The *Geotrichum* genus consists of several species. *Geotrichum candidum*, the best-known species in the genus, is an acid-tolerant filamentous fungus, but classified as a yeast for more than 25 years (Eliskases-Lechner *et al*., 2011).

Geotrichum candidum is usually found in semi-solid or liquid substrates rich in various nutrients, including decomposing plants, effluents from industry and a large variety of foods (Rattray, 2002). *G. candidum* forms smooth, white to cream, velvety, flat colonies, often with a central umbo, on solid media, forms yeast like colonies 7 cm in diameter in 7 days on malt extract agar at 25°C to its rapid growth rate, and forms such peels also on the liquid media (Loo, 2006).

2.2. Taxonomy of the biological yeast like model: *Geotrichum candidum*

Link first described the yeast *Geotrichum candidum* in 1809. Since then, some synonyms have been assigned to it, for example: *Botrytis geotricha* (Link,1824), *Oidium milkis* or

Oospora lactobacilli (Wouters *et al*., 2002) *Geotrichum* species (Butler and Petersen, 1972) *Galactomyces geotrichum* (Redhead and Malloch, 1977), *Galactomyces candidus* ((Hoog and Smith, 2004).

Geotrichum candidum, taxonomically is accepted as the yeast with moldy tendencies by the major taxonomic monography (Koňuchová *et al*., 2016). Its taxonomic classification is as follows ; *Geotrichum candidum in* its asexual form *(teleomorph: Galactomyces candidus* in its sexual form*)* is part of the phylum Ascomycota, in the class Saccharomycetes and in the order Saccharomycetales (Alper *et al*., 2011).

Table 1: *Geotrichum candidum* classification.

Kingdom	**Fungi**
Phylum	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Dipodascaceae
Genus	*Geotrichum*
Species	*Geotrichum candidum*

2.3. Yeast habitats

Geotrichum candidum is a yeast commonly present in the environment and is commonly found in soil, air, water and on various surfaces and in the human respiratory and gastro-intestinal tracts (Alper *et al*., 2011). However, it is best known for its specific habitat in dairy products, particularly in the cheese production process, where it is associated with bloomy rind cheeses, such as Camembert, Brie, and other similar cheeses whose outer surfaces are inoculated with spores of the strain forming a white layer to enhance cheese characteristics which give it specific appearance, taste, and aroma commonly described as earthy, fermented and complex. Overgrowth on the surface of bloomy rind cheeses matured by the fungus results in a slippery rind defect (Eliskases-Lechner *et al*., 2011 and Rattray, 2002).

Beyond food production environments, *Geotrichum candidum* can be found in a variety of habitats, such as: fruit (Yaghmour *et al*., 2012) including grapes , citrus, bananas , tomatoes ,fruit juices (Eliskases-Lechner *et al*., 2011) , cereals (Hattingh *et al*., 2014) including rise grain and bread , plants and vegetables (Splittstoesser *et al*., 1980) as cucumber, helping to break down plant debris. *Geotrichum candidum,* can also be found in aquatic environments, such as lake waters (Vadkertiová and Sláviková, 1995) marine sources (Gad *et al*., 2022).

This species has been isolated in several regions in the world such as : Europe ; France (Marcellino *et al*., 2001) considering the specific geographical origin of bloomy rind cheese production ; Spain (Belén Flórez *et al*., 2007), Germany ,Denmark, Sweden, Netherlands ,Belgium, Puerto Rico, Australia, US, UK, Indonesia, South Africa, Zimbabwe (Gente *et al*., 2006), Canada (Perkins *et al*., 2020) Egypt (Gad *et al*., 2022), India (Mookherjee *et al*., 2018), and Algeria (Chaib *et al*., 2024).

2.4. Yeast characteristics

The characterization of *Geotrichum candidum* requires the description, and identification of several aspects of the fungus. The following are some of the elements that are important in understanding *Geotrichum candidum*.

2.4.1. Morphological characteristics

Geotrichum candidum strains are classified into three types:

Type 1 is made up of, yeast-like strains (a yeast rather than a fungus) with colonies characterized by their creamy color and a great capacity to produce arthrospores (arthroconidia), have slight proteolytic activity and optimal growth at temperatures between 22°C and 25°C (figure 1.A). Type 3 is made up of white strains with true mycelium (a fungus than a yeast), have high proteolytic activity, low production of arthrospores and optimal growth temperatures between 25°C and 30°C (figure 1.B).

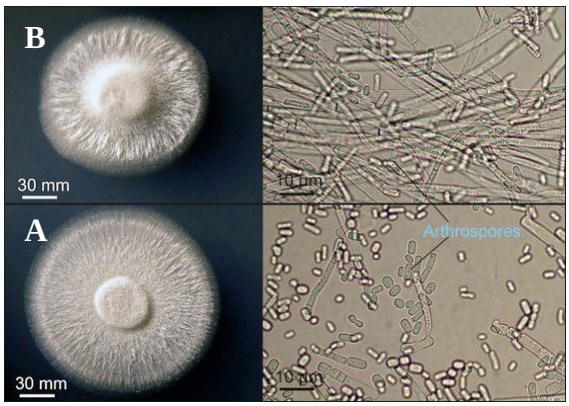

Figure 1: Macroscopic and Microscopic appearance of a *G. candidum* colony grown in a Petri dish in the laboratory (**A:** fungiform strains **B:** yeast-like strains) (Wolf, 2015).

Type 2 is made up of strains that are not clearly classifiable as either type 1 or type 3 (Gueguen and Jacquet, 1982 and Rattray, 2002).

Its morphological appearance resembles that of molds due to the partitioned hyphae formed, which can be broken down into chains or arthroconidia (Figure 2) during the fragmentation process. Typically, they are cylindrical, barrel-shaped or ellipsoidal and usually measure 6-12×3-6 μm (Eliskases-Lechner *et al*., 2011).

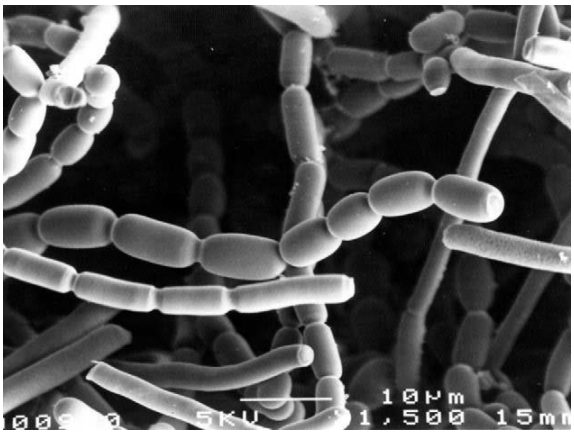

Figure 2: Arthroconidia formed by breaking up of hyphae after 48h in YEG broth (Eliskases-Lechner, 2002).

2.4.2. Growing condition

The presence of yeasts including *Geotrichum candidum* can be influenced by different conditions in the environment, the availability of nutrients and physical factors.

2.4.2.1. Nutritional factors

In general, yeasts grow in both solid and liquid nutrient media that provide dextrose as a carbon source for their energetic metabolism, mineral salts and other essential nutrients that supply nitrogen, phosphorus and trace elements. Yeasts use nitrogen sources such as yeast extract and bactopeptone, which provide many of the metabolites that cells would synthesize during growth under minimal growth conditions (Bergman, 2001). In fact, yeast extract is considered to be the main stimulator of the yeast's microbial growth (Deak, 2006). Commonly used culture media include YPD medium (yeast extract, peptone, dextrose) and Sabouraud medium.

G. candidum has the ability to utilize a variety of carbon sources. It can utilize other amino acids with glucose as a carbon source (Eliskases-Lechner, 2002). Practically all *G. candidum* isolates from the dairy environment are able to assimilate glucose, galactose, sorbose, xylose and glycerol. Lactate is a great source of carbon and energy and its assimilation varies with strain origin, while lactose is not metabolized (Perkins *et al*., 2020). In addition this yeast

species can assimilate sodium succinate and sodium citrate differently extract (Boutrou and Guéguen, 2005).

G. candidum, like other microorganisms, utilizes the organic nitrogen available in simple forms such as peptone and casein or in complex substrates like yeast extract. A few amino acids are used only as nitrogen sources (Glu, Asp, Val, Leu, Pro, Arg, Ala, Lys, Ser, Gly), but when amino acids are used as a carbon source, excess nitrogen is liberated in the form of ammonia as a by-product of the deamination of these nutrients. (Boutrou and Guéguen, 2005).

In contrast to other yeasts, the growth of this species is inhibited by salting. Concentrations of 1% NaCl provide a slight growth suppression. NaCl concentrations of 5-6% have an inhibitory effect. Besides, *G. candidum* has the ability to grow without vitamins (Eliskases-Lechner, 2002).

2.4.2.2. Physical factors

Yeasts' temperature and growth range limits differ from species to species. Mesophilic, developing best at temperatures between 20 and 30°C. Psychrophilic, having a minimum growth temperature as low as -1 to 4°C and a maximum at around 20°C. Only a few yeasts can grow above 40°C, and none can be considered thermophilic. In addition, yeasts can develop in pH ranges between 4.5 and 5.5, and some tolerate a wide range between 3 and 10 (Deak, 2006).

G. candidum is a mesophilic yeast and most studies have appeared that temperatures close to 30 °C are more suitable for its development. its temperature ranged from 5 to 38 °C with an optimum around 25°C with a pH of 5.0 to 5.5 (Eliskases-Lechner, 2002) while the maximum temperature at which it can grow is 35–38°C (Pitt and Hocking, 2009). In contrast, the coldest temperature at which some growth can be observed is 5°C (Hudecová and Liptáková, 2009). These values are common and may change depending on particular developing conditions and the assortment of *Geotrichum candidum* utilized optimal development conditions, include constant moisture content (Featherstone, 2015) and pH ,according to Boutrou and Guéguen, (2005) it’s can grow at different wide pH ranges, from pH 3-11, from pH 3.5 to 9, below pH 4.4, at pH 3.4, and 2.2.

2.4.3. Molecular characteristics

Molecular identification of *G. candidum* is the most rapid and powerful technique used to identify yeasts. It is based on the analysis of DNA or RNA molecules, either at genome level, or by targeting certain well-defined fragments of the yeast. Among the techniques applied are

sequencing of the D1/D2 region of the 26S rRNA gene, which is widely used in identification tests, and/or restriction analysis (PCR-RFLP) of non-coding regions of ribosomal DNA, which includes internal transcribed spaces and 5,8S rDNA (ITS-5,8S region) (Baffi *et al*., 2011).

Recently, MALDI-TOF (Matrix-assisted Laser Desorbtion Ionization-Time of Flight) mass spectrometry has been developed in clinical microbiology for the identification of microorganisms after culture. With this technique, the mass and abundance of proteins between 2.000 and 15.000 Da can be precisely quantified from "intact cells", without prior extraction, in just a few seconds (Alanio, 2013).

2.4.4. Enzymatic activities

As a yeast, *Geotrichum candidum* species strains are able to produce a plethora of biotechnologically important enzymes including cellulases, xylanases, glucanases, lipases, proteases, pectinases α-amylases and chitinases, and which are involved in various metabolic processes.

2.4.4.1. Protease and aminopeptidase activities

Depending on the species studied, *Geotrichum candidum* produces both intracellular and extracellular proteinases, the last mentioned are known to hydrolyze β and α_{s1} caseins (Boutrou *et al*., 2006).

Gueguen and Lenoir (1976) have studied the proteinase activity of *G. candidum*, and have detected extracellular enzyme activity with a pH optimum between 5.5 and 6 and a temperature optimum between 55°C; that of enzymes of intracellular origin also shows maximum activity at a pH of 5.5 to 6 and a temperature optimum between 50°C and 55°C. However, studies have shown that intracellular proteolysis is always greater than extracellular proteolysis (Sacristán *et al*., 2012).

Geotrichum candidum proteases are specifically used in food production, especially in the cheese industry, where they significantly enhance the aromatic complexity and texture of finished products. Muhammad *et al*. (2019) studied the purification of the alkaline thermostable serine protease isolated from *G.candidum* QAUGC01 and which are the main characteristics that qualify this product as an ideal candidate for industrial application.

G. candidum also produces an extracellular aminopeptidase. These enzymes are used to break down hydrophobic peptides of low metabolic weight, which can then be used as sources of carbon and nitrogen (Sacristán *et al*., 2012).

2.4.4.2. Lipase activity

The lipase from *G candidum* is interesting because of its unique selectivity for long-chain fatty acids with cis-9 double bonds, such as oleic and linoleic acids, or other fatty acids palmitic acid and stearic acid depending on the individual, it has a unique specificity for unsaturated fatty acids, and esters of these fatty acids are hydrolyzed more rapidly than most others (Burkert *et al*., 2005 and Loo, 2006). It produces two extracellular lipases, lipase A and lipase B with molecular mass 58–66 kDa (Shimada *et al*., 1989, 1990) or from 50 to 62 kD (Bertolini *et al*., 1994 and Sidebottom *et al*., 1991) depending on the individual. There are many reports in literature about production and application of these lipases. Brabcová *et al*. (2013) have investigated the purification and the characterization of the two lipases types from *Geotrichum candidum* 4013 with a new lipase (*lip 3*) which was confirmed by Edman degradation procedure and their biotechnological applications.

The effect of *G. candidum's* lipolytic action has been described as having a significant impact on cheese ripening, particularly in terms of aroma development, thus influencing its final texture. These include the typical Camembert (Boutrou and Guéguen, 2005 and Maldonado, 2017).

2.4.4.3. Amylase activity

Although *Geotrichum candidum* is not known for its ability to degrade starch, some strains may exhibit amylolytic activities. A study conducted by Attanayaka *et al*. (2009) on the identification and the purification of α-amylase in isolated *G. candidum* CMSS06 that hydrolyzes raw starch, has shown the potential to be used in the bioconversion of crude starch into low-molecular-weight sugars for alcoholic fermentation, helping the global search for sustainable energy resources. In addition, Divya and Padma (2014) have shown the significance of *Geotrichum* sp. isolate and its ability to produce good cold-active amylase commercially at room temperature (20°- 25°C) and use them for clarifying cold-stored fruit juices in order to maintain juice organoleptic properties and enhance its shelf life. Also, Falih' s (1998) study clearly shows that *G. candidum* soil yeast can achieve substantial yields of amylase.

2.4.4.4. Cellulase and xylanase activities

Despite some research into the production of cellulase and xylanase from *G. candidum*, certain strains of the species have the capacity to exert cellulolytic activities, its produces two types of endocellulase, endocellulase I and endocellulase II, with molecular masses ranging from 63 to 130 kDa. Both enzymes are stable at 55°C. Endocellulase I was shown to be stable between pH 3.5 and 7.0, and endocellulase II was stable between pH 3.0 and 7.5 following

standing at 4°C for 24 hours at different pHs. Endocellulase I clearly favored the enzymatic hydrolysis of crystalline cellulose more significantly than the ordinary type of endocellulase, playing an influential role in the first stage of hydrolysis of native cellulose and making the fibrils more amenable to cellobiohydrolase (Mo and Hayashida, 1988). Most extracellular cellulolytic enzymes in *Geotrichum candidum* from *Geotrichum candidum* 3C, such as cellobiohydrolases, endo-1,4-β-gluconases, β-glucosidases, arabinofuranosidases and polygalacturonases, were obtained in high-purity compositions by Rodionova *et al*. (2000). *G. candidum* cellulase is known as an alternative renewable energy resource, and is used in biological processes to produce ethanol from lignocellulose biomass, in particular rice straw. This solves the high cost and hostile environment of gas (Gad *et al*., 2022).

On top of that this species, in particular *Geotrichum candidum* 3C has shown its ability to produce three endoxylanases types, endoxylanase I, which attaches to cellulose, endoxylanase II, which attaches to insoluble xylan, and endoxylanase III, which cannot attach to dissolvable substrates (Rodionova *et al*., 2002). In Addition to the main application of xylanase in the pulp bleaching process, other applications of xylanase in the food and feed industry, bread quality improvement, agricultural waste treatment, food processing plants, biofuels, degumming, seed germination, xylooligosaccharide (XOs) production and bioenergy. Seed germination, xylooligosaccharide (XOs) production and bioenergy have been described by the Goswami and Rawat (2015).

2.4.4.5. Pectinase activity

Production of pectinase is not commonly carried out in yeasts. *Geotrichum candidum* AA15 has been characterized and shown to be optimally active at pH 5.5 and 50°C, and stable at a wide pH range and moderate temperatures. It is very specific towards pectin, which it breaks down efficiently into smaller molecules. Pectinase demonstrated significant potential application in orange juice clarification, effectively reducing cloudiness and viscosity. It also enhanced juice clarity and performance, which made it suitable for industrial use in the juice processing sector (Ahmed, 2020).

2.5. *Geotrichum candidum* biotechnology applications

Geotrichum candidum's enzyme profile can vary depending on specific strains, growing conditions and environments (Piegza *et al*., 2014). Below is an overview of the enzymes that can be produced by this species (Table 2).

2.5.1. Cheese industry

G. candidum is a major factor in industrial cheese processing, and it's considered a mycological mystery for its ability to produce the lipases and proteases, as well as catabolism of amino acids and free fatty acids, and deacidification activity are of primary importance for their use in cheese industry that are responsible for the specific flavors and aromas of cheese (Eliskases-Lechner *et al*., 2011).

2.5.2. Malting

This strain is also suggested as a starter culture in malt production, increasing soluble nitrogen quality, grain fragility, Kolboch index and reducing β-glucan levels and viscosity in the final product (Piegza *et al*., 2014). In addition, it has been demonstrated to reduce toxic metabolites that affect the quality and safety of raw materials and industrial final products, due to its high production of phenyllactic acid (Kawtharani *et al*., 2020 and 2022).

2.5.3. Probiotic strain

G. candidum is used as a nutritional probiotic in fish, focusing on their growth rates, intestinal enzyme activity, and immune system function (Ibrar *et al*., 2017 and Kamilari *et al*., 2023).

2.5.4. Antimicrobial activity

G. candidum produces a variety of antimicrobial compounds, including doleacetic acid, phenyllactic acid and phenylethanol and their derivatives, as well as peptides. Phenyllactic acid has extensive antibacterial effects against bacteria, yeasts and molds, causing cell death. Phenylethyl alcohol affects the plasma membrane and the transport systems for sugars and amino acids. Indoleactic acid and phenyllactic acid inhibit the growth of *Listeria monocytogenes* and induce structural changes. Aromatic amino acids influence the synthesis of antimicrobial compounds in certain isolates (Kamilari *et al*., 2023).

2.5.5. Production of enzymes with industrial interest

G. candidum specie have the ability to produce a wide range of enzymes qualify it for a batch of important industrial applications. α-Amylase in the *G. candidum* species isolated by Attanayaka *et al*.(2009) has revealed the possibility of being applied in the bioconversion of raw starch into low-molecular-weight sugars for alcoholic fermentation contributing to the worldwide search for renewable energy resources. Its lytic polysaccharide monooxygenases (LPMOs) are particularly interesting candidates for enzymatic combinations applied to the improvement of biorefineries (Ladevèze *et al*., 2017). While, its cellulase has shown great

success in the carboxymethylcellulose salt degradation from agricultural waste (rice straw), the fermentation of which has led to the production of bioethanol (Gad *et al*., 2022).

Its lipases have numerous industrial applications, including pharmaceutical and cosmetics production, agrochemicals, surfactants, biolubricants, waste disposal and influencing the sensory characteristics of food products and detergent industries (Kamilari *et al*., 2023). The alkaline proteases of this strain are of high biotechnological value for their many applications in the food, pharmaceutical and textile industries, as well as in silver recovery, detergent and waste treatment, and the resolution of amino acids (Agrawal *et al*., 2004 and Kamilari *et al*., 2023).

The pectinases produced by *Geotrichum* spp. such as *G. candidum* AA15, are considered good candidates for use by the fruit juice industry as described by Ahmed (2020) , since pectin is a molecule that rounds out cloudy juices with a colloid texture, making the addition of this enzyme an excellent choice for juice clarification.

Several enzymes such as aldehyde dehydrogenases, glutamate dehydrogenases, and Baeyer–Villiger monooxygenases, those associated with oxidation reactions have been used by the pharmaceutical and chemical industries for the oxidation of alcohols, sulfides, aldehydes, Baeyer-Villiger oxidation, and hydroxylation, has been demonstrated produced by *G. candidum* indivudus (Hollmann *et al*., 2011 and Zhang *et al*., 2017).

2.5.6. Environment biotechnological applications

In terms of bioremediation, *candidum* sp. shows promise in reducing pollution by decomposing organic waste and phenolic compounds, with the ability to remove pollutants such as heavy metals from the aquatic environment, also the ability to degrade glycerol trinitrate, a compound commonly found in explosives (Kamilari *et al*., 2023).

Table 2: Industrial enzymes produced by *G .candidum* species.

Enzyme	*G. candidum* individuals	Industrial application	Reference
Amylase	*G. candidum* CMSS06	Renewable energy production	(Attanayaka *et al*., 2009)
Lipase	*G. candidum* NRRLY-552	Food industry	(Santos, 2021)
Cellulase	*G. candidum* Strain Gad1	Bioethanol production	(Gad *et al*., 2022)
Pectinase	*G. candidum* AA15	Fruit juice industry	(Ahmed, 2020)
Protease	*G. candidum* QAUGC01	Dairy food	(Muhammad *et al*., 2019)
Lytic Polysaccharide Monooxygenases	*G. candidum* AA9	Biorefineries enrichment	(Ladevèze *et al*., 2017)

3. Amylase

3.1. Definition, classification and nomenclature

α-Amylase which is a hydrolase enzyme that breaks the α-1,4 osidic bonds of polysaccharides such as starch, releasing glucose, maltose and maltodextrin, in the CAZy database classified as a part of the glycoside hydrolase families 13 (GH13 families).There are 2 types of hydrolases : endohydrolases, which act on the substrate molecule, and exohydrolases, which act on the non-reducing terminal ends, α-amylase as well as known as endoamylase, acts on starch and catalyses its hydrolysis into shorter oligosaccharides by cleaving the α-D-(1-4) glycosidic bond present in the inner part of amylose and amylopectin chain (Sundarram and Murthy, 2014 and Tiwari *et al*., 2015)**.**

Amylose and amylopectin have different structures and properties; Amylose is composed of starch molecule representing 20 to 25% of the starch molecule (Figure 3.A) composed of 6000 glucose units. Amylopectin represents 75 to 85% of starch and is composed of branched chains of glucose units. It is formed of around two million glucose units. The branches of the amylopectin side chain are made up of around 15 to 45 glucose units attached by α→1,6 bonds, and the linear chains of 10 to 60 glucose units by short α-1,4 li bonds (Figure 3.B) (Souza and Magalhães, 2010 and Sundarram and Murthy, 2014).

According to the International Union of Biochemistry and Molecular Biology (IUBMB), for enzyme nomenclature, EC 3.2.1.1 is the commission number of the enzyme and α-1,4- glucan-4-glucanohydrolase is the scientific name of α-amylase. Other names have been mentioned such as glycogenase; endoamylase; Taka-amylase A.

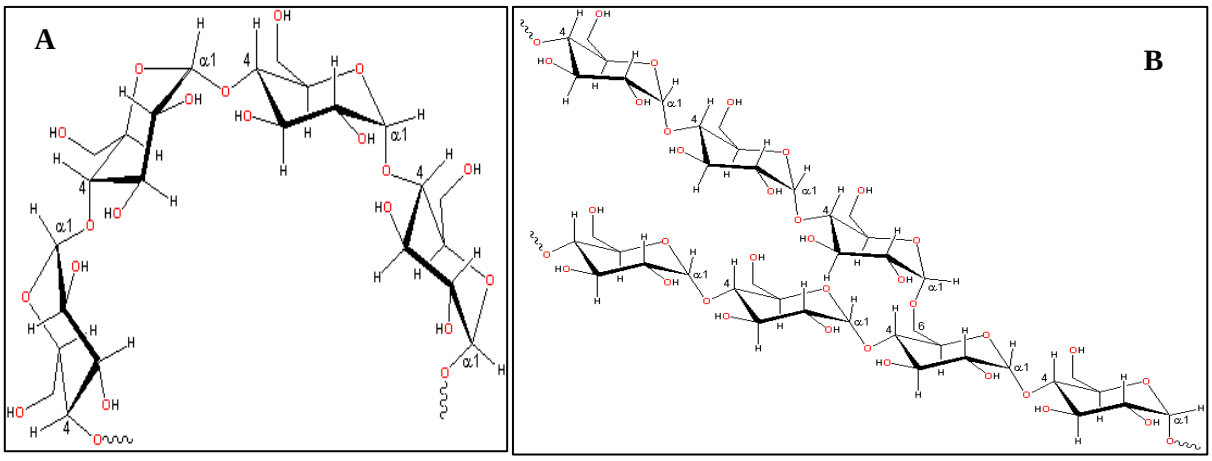

Figure 3: Starch structure

(A: Amylose**, B:** Amylopectin**)** (Rosliza and wan nik, 2010)**.**

3.2. Structural and characteristics of α-amylase

α-Amylase is formed by a single oligosaccharide chain containing around 512 amino acids, it is composed of three domains A , B and C (Sahni *et al*., 2015) and its structure is shown in Figure 4. The A domain is the largest, presenting a typical barrel shaped (β/α) 8 super structure. This barrel (β/α) 8 also known as the TIM barrel, which contain the residuals from the catalytic site and include four highly conserved regions in their primary sugar syrups, The B domain is the smallest, is positioned between the A and C domains and is linked to the A domain by a disulfide bond. It serves to form a calcium-binding site against the wall of the beta-barrel of domain A. Domain C has a β sheet structure linked to the A domain by a single polypeptide chain and appears to be an independent domain of unknown function. The active (substrate-binding) site of amylase is located in a wide cleft between the carboxyl ends of the A and B domains. Most of the a-amylases are metalloenzymes, which require calcium ions (Ca^{2}+) for their activity, structural integrity and stability (Muralikrishna and Nirmala, 2005 and Afzaljavan and Mobini-Dehkordi, 2012).

As all enzymes, α-amylase is glycoprotein generally monomeric in structure, such alpha-amylase, MalS type from *Escherichia coli* (Spiess *et al.*, 1997) , α-amylase AmyC from *Thermotoga maritima* (Dickmanns *et al*., 2006), but also tetrameric or dimeric (Kennedy, 1988). The glycosylated part protects enzymes against denaturation and proteolysis (Zhou and Qiu, 2019).

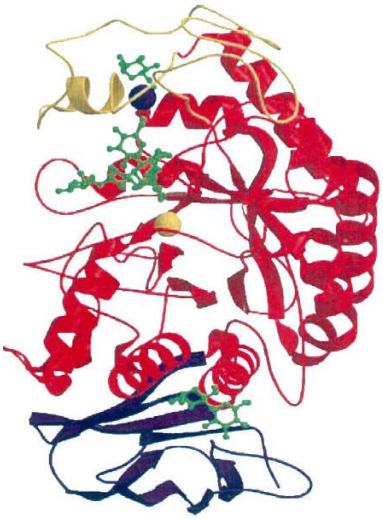

Figure 4: The 3D structure of α-amylase (Souza and Magalhães, 2010).

(**Red**: Domain A, **Yellow**: domain B, **Purple**: Domain C, **Blue sphere**: the calcium ion localized in catalytic center, Yellow **sphere:** the chloride ion localized in catalytic center, **Green**: structures that are linked to the active site and surface binding sites).

3.3. α-Amylase mode action

Generally, endogenously acting amylases that hydrolyze α- (1-4) glycosidic bonds of starch polymers internally. Different models of amylase action have been proposed, such as random action and multiple attack action (Figure 5).

In random action, the polymer molecule is hydrolyzed successively and completely before the enzyme-substrate complex is dissociated. In the second case, a single bond will be hydrolyzed per effective interaction, it has also has been referred to as a single or multi-string attack action. Multiple attack action is an intermediary between single-chain and multiple-chain action, in which the enzyme successively breaks multiple glycosidic bonds after the first (random) hydrolytic attack, before dissociation (Tiwari *et al*., 2015).

The majority of endoamylases, however, have a low to very low level of multiple attack action which increases with temperature to a degree dependent on the amylase itself (Tiwari *et al.,* 2015).

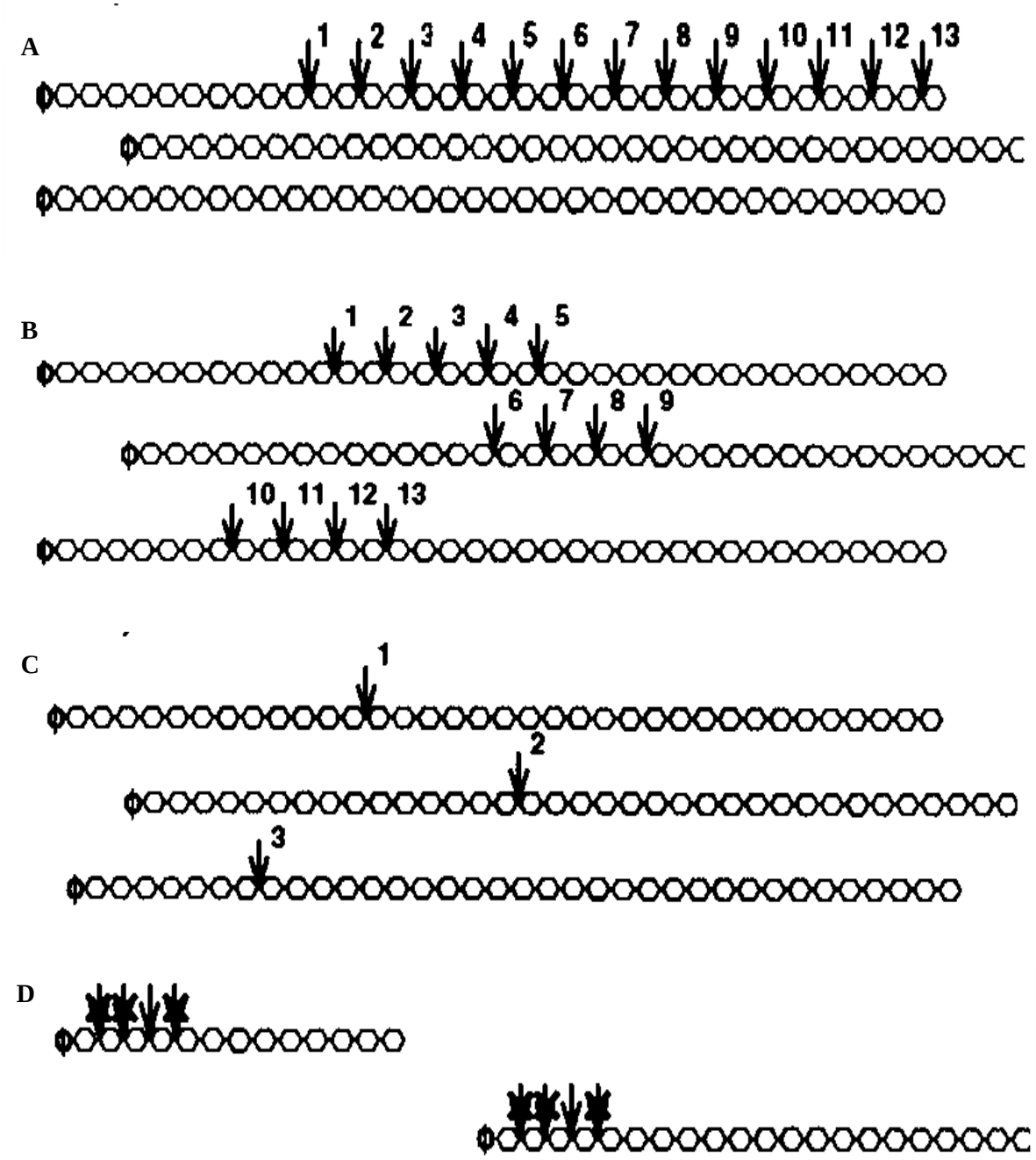

Figure 5: Modes of action of amylase on amylose and amylose fragments (**A:** Single chain attack**, B:** Multiple attack**, C:** Multiple chain attack**, D:** Preferred attack**)** (Bijttebier *et al*., 2008).

3.4. α-Amylase sources

Alpha-amylase is isolated from plants, animals, and microorganisms (Muralikrishna and Nirmala, 2005). Applications in industrial sectors have been dominated by enzymes from microbial sources, in particular, due to the economic capacity for mass production and the fact that microbes are easy to manipulate, amylases with desired characteristics can be obtained (Afzaljavan and Mobini-Dehkordi, 2012). Fungi had been found to be good source for amylases enzymes; they are primarily confined to *Aspergillus* sp. *Penicillium* sp. which have the most a significant industrial importance (Saranraj and Stella, 2013). In addition, diverse range of bacteria utilizes extracellular or intracellular enzymes capable to transforming starch or glycogen into energy and carbon sources. In commercial applications, α-amylase is predominantly sourced from the *Bacillus* genus. Approximately half of the worldwide enzyme market is believed to be comprised of enzymes derived from *Bacillus* species (Afzaljavan and Mobini-Dehkordi, 2012).

3.4.1. Amylolytic yeast

Traditionally, yeasts play a key role in various food fermentations and the production of items such as beers, ciders, wines, sake, baked goods, cheese, sausages, and other fermented foods. In the past few years, yeasts have gained prominence as a significant enzyme source, alongside their notable lipid production, short fermentation cycles, ability to thrive in diverse climates, tolerance to low pH levels, and capacity to grow on a wide range of substrates (Ramachandran *et al*., 2004). Yeasts exhibiting enzyme production, including α-amylase, lipase, cellulase, and esterase, are ubiquitously present in nature (Okpara, 2022).

As mentioned by Djekrif-Dakhmouche *et al.* (2016), the number of yeast-derived extracellular amylases is limited. The documented examples include *Cryptococcus heimaeyensis* HA7, *Trichosporon pullulans, Saccharomycopsis bispora, Saccharomycopsis capsularis, Saccharomycopsis fibuligera (Endomycopsis fibuligera), Lipomyces starkeyi* NCYC 1436, *Candida* sp., *and Candida parapsilosis, Candida glabrata, Rhodotorula mucilaginosa.*

3.5. α-Amylase production

Commercial-scale production of α-amylase primarily relies on two methods: submerged fermentation and solid-state fermentation. The former, a more traditional approach, has been employed for enzyme production from microbes for a considerable period. The latter, solid-state fermentation is a relatively newer method. Both methods are influenced by various

physicochemical factors, including pH, temperature, aeration, oxygen transfer, and moisture (Souza and Magalhães, 2010).

3.5.1. Submerged fermentation

The process of submerged fermentation involves the cultivation of microorganisms in a liquid nutrient broth, facilitating the production of industrial enzymes and other metabolites. In this method, selected microorganisms like bacteria, yeasts, and fungi are cultured in enclosed vessels filled with a nutrient-rich broth, also known as the fermentation medium. Within this medium, the microorganisms digest the nutrients, leading to the release of the desired enzymes into the cultivation solution (Prado Barragán *et al*., 2016), offering advantages such as easy obtaining of products, speed of substrate consumption, facilitates sterilization and purification processes (Farooq *et al*., 2021).

This approach also provides an opportunity to simplify the cultivation of genetically modified bacteria (Paulová and Brányik, 2013) .

3.5.2. Solid state fermentation

Solid-state fermentation (SSF) has garnered recognition in the biotech industry in recent years, primarily for its potential in generating biologically active secondary metabolites. It stands as an attractive substitute for submerged fermentation, offering advantages such as enhancing productivity, a simplified technique, reducing capital investment, lower energy requirements, minimal water output, improving product recovery, and the absence of foam build-up enhanced productivity, and is applicable across various sectors, encompassing the production of feed, fuel, food, industrial chemicals, and pharmaceutical products (Singhania *et al*., 2009 and Souza and Magalhães, 2010).

According to the theoretical concept of water activity, fungi and yeast have been identified as appropriate microorganisms for solid-state fermentation (SSF), while bacteria are generally deemed unsuitable for this process (Pandey, 2003).

3.5.3. Substrates

3.5.3.1. Carbon sources

Various carbon sources are employed in α-amylase production, include glucose, lactose, galactose, fructose, sucrose, date syrup, starch, molasses (Simair *et al*., 2017), xylose, and maltose (Elmansy *et al*., 2018). However, due to their high cost, there is a potential for substitution with more economical agro-wastes with the intention of rendering the enzyme production process more environmentally friendly. Food and agricultural waste can be utilized

as substrates for the production of a diverse range of fermented products and enzymes (Sundarram and Murthy, 2014).

Various agricultural substrates have been used as alternative carbon sources such as cassava, corn, taro, purple sweet potato, potato, breadfruit, canna, gembili, gadung and sago by Saryono *et al*. (2023), and potato starch, cassava starch, rice flour, corn starch, glutinous rice flour, and wheat flour by Phonlamai *et al*. (2024) . In addition, several vegetable and plant wastes, notably corn cobs (CC), onion peels (OP), rice straw (RS), potato shells (PS), Molokhia stem (MS), okra suppression (OS), lemon peels (LP) and pea peels (PP), have been tested and approved for amylase production by *Bacillus* spp. (Mostafa *et al*., 2024).

On top of that, one significant substrate in this category is oil cake, a by-product of oil extraction. The chemical composition of oil cake varies depending on the extraction methods employed but generally contains a substantial amount of carbohydrates. coconut oil cake, sesame oil cake, groundnut oil cake, palm kernel cake and olive oil cake have been used previously for amylase production (Balakrishnan *et al*., 2021 and Ramachandran *et al*., 2004).

3.5.3.2. Nitrogen source

The choice of nitrogen source depends on the specific microorganisms used. Most commonly used organic sources of nitrogen include peptone ,beef extract , trypton, yeast extract (Samanta and Jana, 2024), malt extract (Shad *et al*., 2023), and corn steep liquor (Omidiji *et al*.,1997), and few inorganic sources of nitrogen are ammonium hydrogen phosphate, ammonium sulfate, and ammonium chloride (Jujjavarapu and Dhagat, 2018).

3.6. Optimization of α-amylase production

In order to optimize enzyme production, two principal approaches are proposed: recombination techniques adapted to increasing amylase production, and the enhancement of culture conditions.

3.6.1. Recombinant techniques for enhanced amylase production

The production of amylase through recombinant DNA technology entails selecting a potent amylase gene, inserting the gene into a suitable vector system, transforming it into an efficient bacterial system to generate a substantial amount of recombinant protein (with the aid of an expression-vector promoter-inducing agent), and subsequently purifying the protein for downstream applications (Gopinath *et al*., 2017).

To enhance alpha-amylase production, the approach involves amplifying the copy number of genes for a higher amylase yield and screening mutant libraries to select the most efficient mutant strain. This is achieved by introducing host DNA or plasmid into new bacterial cells, where the bacterial cells overexpress amylase mRNA, resulting in an increased production of the enzyme (Jujjavarapu and Dhagat, 2018).

3.6.2. Optimization of cultural conditions

The refinement of fermentation conditions, especially the optimization of physical and chemical parameters, holds significance in the advancement of fermentation processes. This is attributed to the substantial influence these conditions have on the economic viability and practicality of the process.

3.6.3. One Factor At Time

It is a prevalent technique for optimizing fermentation medium components and conditions, this method involves keeping all parameters constant except for one independent variable with a singular impact on medium components and fermentation conditions. While this approach is straightforward and uncomplicated, it is time-consuming, does not explore the interactions between variables, and can be costly, particularly when conducting a large number of experiments (Abdel-Rahman *et al*., 2020).

3.6.4. Placket and Burman design

A statistical experimental design, specifically a two-level fractional factorial design, is designed to pinpoint crucial physicochemical parameters among N variables using N+1 experiments, without explicitly considering interaction effects between the variables. This method, capable of examining up to 99 factors with 100 experimental runs, plays a significant role in advancing fermentation bioprocess development. It efficiently screens the main factors of interest and subsequently optimizes them to enhance industrial process performance (Ekpenyong *et al*., 2017 and Humbird and Fei, 2016).

3.6.5. Response Surface Methodology (RSM)

Response Surface Methodology (RSM), pioneered by Box and Wilson, it comprises a set of mathematical and statistical techniques designed to analyze problems, such as the one presented, through an empirical model (Sarabia *et al*., 2020) .It is particularly serves as a suitable design model to elucidate the combined effects and study multiple factors influencing fermentation responses by varying them in a limited number of experiments. They are more effective than “one-factor-at-a-time” approach in screening interactions between different

variables and describing the role of the interactions of each component in the process ultimately identifying the optimal conditions to maximize enzyme production (Abdel-Rahman *et al*., 2020 and Mostafa *et al*., 2024).

3.7. Purification

Purifying enzymes, as a general practice, is challenging and typically involves intricate procedures with multiple processing steps to yield limited quantities of pure enzymes. Various techniques have been explored in the past for enzyme separation, including methods such as ammonium sulfate precipitation, ultrafiltration, three-phase partitioning, aqueous two-phase extraction, ion exchange, gel filtration, affinity chromatography, expanded bed chromatography, polyacrylamide gel electrophoresis, and chromate-focusing (Singh Kim *et al*., 2022). Strategies implemented for the purification of microbial α-amylases in recent times are presented in the Table 3.

The initial amylase enzyme, in its crude form, can be precipitated and concentrated through methods such as ammonium sulfate precipitation or the use of organic solvents. Subsequently, the precipitated sample can undergo dialysis against water or a buffer to achieve additional concentration, as suggested by reference (Owolabi *et al*., 2023). Following this, various chromatographic techniques, including ion exchange, gel filtration, and affinity chromatography, can be employed for further separation and purification of the enzyme.

The utilization of Stirred Cell for ultrafiltration membrane processes stands out as a highly appealing method for enzyme separation. This preference arises from the selectivity of the process, its gentle treatment of the enzyme, and the diverse range of custom-manufactured membranes that are accessible. This approach holds promise as an attractive alternative for enzyme purification. It's noteworthy that limited research has been conducted on the separation of proteins within a crude mixture using ultrafiltration, as indicated by the work of Slater *et al*. in 1986.

Table 3: Purification strategies for microbial alpha amylase.

Organism	Purification method	References
Bacillus cereus	ASP , Dialysis, OSP	(Abo-Kamer *et al*., 2023)
***Pseudomonas balearica* VITPS19**	ASP ,Dialysis , GFC	(Kizhakedathil and C, 2021)
Aspergillus flavus	ASP, Dialysis, AEC,GFC	(Owolabi *et al*., 2023)
***Rhizopus oryzae* FSIS4**	TPP	(Ait Kaki - El-Hadef El-Okki *et al*., 2017)
***Clavispora lusitaniae* ABS7**	OSP, GFC,AEC	(Dakhmouche Djekrif *et al*., 2021)
***Aspergillus flavus* S2-OY**	ASP, Dialysis ,AEC	(Oyedeji *et al*., 2023)
***Aspergillus tamarii* MTCC5152**	SAP, Dialysis, AEC, GFC	(Arunachallam *et al*., 2023)
***Anoxybacillus* sp. YIM 342**	Ultrafiltration , ASP , GFC	(Zhang *et al*., 2015)
***Haloterrigena turkmenica* DSM-5511**	Ultrafiltration, GFC	(Santorelli *et al*., 2016)
***Anoxybacillus thermarum* A4 strain**	ASP, Ultrafiltration with Amicon filter, GFC	(Baltas *et al*., 2016)
***Geotrichum candidum* CMSS06**	ASP,Dialysis,AEC	(Attanayaka *et al*., 2009)

Abbreviations: ASP: Ammonium sulfate precipitation, OSP: organic solvent precipitation, GFC: Gel filtration chromatography, AEC: Anion exchange chromatography, TPP: Three-phase partitioning.

3.8. α-Amylase characteristics

After the purification of the enzyme, the subsequent step involves conducting a characterization of the enzyme. The increasing industrial demand for thermostable alpha-amylase has led to a steady increase in research efforts aimed at discovering new enzyme variants that exhibit enhanced thermal and pH stability along with low molecular weight. Small enzymes offer ease of modification, providing advantages in processes such as immobilization or genetic manipulation, ultimately contributing to increased enzyme selectivity. *Bacillus* species are the major producers of alpha-amylases, with those that exhibit thermostability mostly originating from *Bacillus* or *Geobacillus* (Sudan *et al*., 2018 and Febriani *et al.*, 2019). The molecular weights of α-amylases range from 10 kDa to 210 kDa, as reported by El-Enshasy *et al*. 2013 . The enzyme with the lowest molecular weight (10 kDa) was extracted from *Bacillus caldolyticus* while the highest molecular weight recorded was 210 kDa from *Chloroflexus aurantiacus* (Mehta and Satyanarayana, 2016). Thermostable α-amylases produced by microorganisms typically exhibit molecular weights between 21 and 160 kDa and are most effective under acidic pH conditions. It should be noted that a low molecular weight alpha-amylase with activity under alkaline pH conditions is rarely reported (Mehta and Satyanarayana, 2016).

α-Amylases are categorized as metalloenzymes. Within this group, many enzymes contain divalent cations, and these ions play crucial roles in activating and stabilizing the enzyme. Among the various divalent cations investigated, calcium and barium have been identified as the most effective for amylase. The stability and activity of bacterial α-amylase are controlled by calcium ions, as each enzyme binds three Ca^{2+} and one Na^{+} per subunit. The CaI, a typical calcium-binding site, is situated in the interface domain A and B, closely positioned to the active site (El-Enshasy *et al*., 2013). Various metal ions, including Na^{+}, Ca^{2+}, K^{+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Pb^{2+}, Co^{2+}, Fe^{3+}, Cu^{2+}, exert a significant influence on the activity of α-amylase depending on species (Arunachallam *et al*. and Oyedeji *et al*., 2023).

3.9. Industrial application of amylases

α-Amylase is increasingly recognized for its ability to hydrolyze starch and the versatile applications stemming from this property. This enzyme holds significant potential and finds widespread use in various industrial applications. Enzymes, particularly α-amylase, have replaced conventional chemical hydrolysis methods in diverse industrial sectors, contributing to environmentally friendly processes (Sundarram and Murthy, 2014). The industrial applications of α-amylase are detailed in Table 4.

Table 4: Applications of amylases in various industries.

Industry	Application	Reference
Food	In bakery to improve bread quality	(Ait Kaki - El-Hadef El-Okki *et al*., 2017)
Textiles	Weaving process to remove starch	(Mojsov *et al*., 2018)
Production of fuel alcohol	Bioconversion of starch into ethanol	(Toksoy Oner, 2006)
Pharmaceuticals	The amylase will enzymatically degrade the cross-linked starch, facilitating the release of the drug	(Jujjavarapu and Dhagat, 2018)
Detergents	Detergent additive for laundry.	(Dakhmouche Djekrif *et al*., 2021)
Paper and pulp	Removing starch after coating and surface sizing	(Lee *et al*., 2002)
Biorefinery	Starch liquefaction process	(Arif *et al*., 2019)
Starch conversion	The breakdown of starch into dextrins leads to the creation of a starch suspension with lower viscosity.	(Souza and Magalhães, 2010)

Materials and methods

Materials and methods

Part 1: Isolation and identification of an amylolytic yeast

1. Sampling

Samples were collected using a sterile spatula and placed in pre-sterilized bottles, stored in a cool (4°C) environment until use. The pH of each sample was measured using a laboratory pH meter, and samples were labeled alphabetically for identification.

1.1. Olive pomace (OP) and its rinse water (RW)

Samples were gathered in November 2020 from the Boulahrouf oil mill in the Mila region. They were carefully placed in sterile containers and stored in a refrigerator before being transported to the laboratory. This particular waste material originates from the pressing of Sigoise olives at 60% maturity, characterized by their black, purple, and yellow hues. Additionally, its rinse water utilized during the two rinsing phases is included in the collected samples.

1.2. Mastic oil cake (MC) (*Pistacia lentiscus L*)

Under aseptic conditions, these samples were transported to the laboratory in December 2020 from an oil mill located in the Skikda region.

1.3. Smen (VS)

The Smen used is 100% vegetable Smen of the CEVITAL (Medina) brand, made from refined vegetable oils and fats (palm, copra) as well as certain food additives: ghee flavor, dl-alpha-tocopherol, antioxidant, beta-carotene, color and vitamins A, D, E.

1.4. Olive forest soil (OS)

Soil samples obtained from an olive grove in January 2021 in the Skikda region were taken using the method of Pochon and Tardieu (1962). About 150 g of soil were taken using a sterile spatula after removing the top five centimeters of the surface layer of the soil, then placed in a sterile jar and kept in a cool place (4°C) until use (Ambehabati *et al*., 2020).

1.5. Thermal water (TW)

A sample of thermal water was taken in January 2021 at Hammam El Dabbagh or Hammam El Shallala in the wilaya of Guelma. The sample was taken exactly from the waterfalls where the temperature reaches 95°C in a sterilized glass bottle.

2. Isolation

The procedure involved the use of the successive dilution technique, as described by Zaky *et al*. (2016). In this method, the stock solution was prepared by suspending 1 g or 1 ml of the sample in 9 ml of sterile distilled water, followed by shaking for 5 to 10 minutes. Subsequent dilutions were prepared, reaching up to 10^{-6} for olive pomace and its rinse water, mastic oil cake waste, and smen, and 10^{-9} for contaminated soil. These dilutions were uniformly spread in 0.1 ml increments using a rake on YPGA medium (see Annex 1), as detailed by Salah *et al*. (2021) . Petri dishes were then incubated at 28°C for a period of 3 to 7 days.

Isolation of thermal water is performed by the enrichment technique (Vyas and Chhabra, 2017) consisting of adding 2 ml of thermal water to an Erlenmeyer flask containing 50 ml of YM medium and incubated at 30°C on a rotary shaker at 150 rpm for 24 hours, then yeast colonies were isolated on SDA, YGA, YMA media (Annex 1).

3. Purification

After macroscopic and microscopic observation, well-isolated colonies were purified on YMA medium by successive subculturing. Incubation was carried out at 28°C for 2 to 3 days.

4. Conservation

First batch is preserved in inclined agar tubes, by subculturing the strains on SDA inclined agar tubes with chloramphenicol and ampicillin, the cultures are then incubated during 3 to 5 days for a maximum growth, and then stored at 4°C.

Another batch was stored in Eppendorf tubes. This method involves taking fresh cultures and inoculating them on YM liquid medium with added 30% glycerol, then storing them at -20°C. This batch of strains is viable for 1 to 2 years.

5. Determining the pH of samples

The method consists of using a pH meter to measure the pH value of a solution of 10 g of soil in 90 ml of distilled water and measuring the stock solutions used for isolation, as well as the thermal water.

6. Screening of the enzymatic activities of yeast isolates

6.1. Amylolytic activity

All the yeast strains isolated were tested for their ability to produce alpha-amylase. The screening was carried out on the YPSA medium (Annex 1), using starch as the only carbon

source (Lee et 2011) which was revealed after the addition of a Lugol solution, that gave a clear zone compared to the blue zones containing non-hydrolyzed starch.

6.2. Cellulolytic activity

The cultures were screened for their ability to hydrolyze carboxymethyl cellulose (CMC) on YPCA medium (Annex 1) using the streak method with four quadrant isolates (Buzzini and Martini, 2002). After cell growth, the plates were covered with 1% Congo red solution for 20 minutes. Successive rinses with a 2N NaCl solution removed excess dye. Cellulolytic activity was determined by observing a pale-yellow color around the yeast colonies.

6.3. Esterase activity

Esterase activity on Tween-80 agar medium was assessed by standardizing all strains (4 McFarland) in sterile distilled water. A volume of 62 µL of the yeast suspension was inoculated into 6 mm wells on Tween 80 agar medium following the protocol outlined by Buzzini and Martini (2002) and Riyadi *et al*. (2017). Incubation of the inoculated plates was carried out at 28°C for 72 hours. The presence of a clear zone in the form of crystals around the colony on the TPA medium indicated esterase or lipase production.

6.4. Lipase activity on olive

All Strains are incubated on an olive oil agar medium using red phenol as a pH indicator (Singh *et al.*, 2006 and Lanka and B., 2018)**.** The presence of lipolytic activity is indicated by the development of a yellow zone. Clearing zones were categorized as weak (+) for measurements <20 mm, moderate (++) for 20-30 mm, and high (+++) for zones exceeding 30 mm.

7. Enzymatic index

The enzymatic activity index is a useful indicator for selecting and comparing the enzyme production of different microbial isolates. It is calculated as the relationship between the size of the halo and the degradation capacity of the micro-organisms using the following formula (Carrim *et al*., 2006):

$$EI = \frac{Turbid\ Zone\ diameter\ (mm)}{Colony\ diameter\ (mm)}$$

8. Identification of the selected strain

8.1. Morphological identification

The macroscopic characteristics of the selected strain are noted, based on speed of growth, macroscopic appearance, color, texture, appearance, etc. Microscopic examination is based on the shape and mode of reproduction of the cells. The examination is carried out in the fresh state (magnification X 40 and X 100) of prepared smears from fresh cultures in YPDA (Sacristán *et al*., 2012).

8.2. Molecular identification

Genotype identification of the selected strain is based on amplification of the D1/D2 region of the 26S ribosomal RNA gene (26S rRNA), which, together with the 18S and 5.8S subunits, forms a transcription unit repeated in tandem around 100 times. Each transcription unit is separated from the next by an IGS (Inter Genic Spacer), the length of which varies from species to species

8.2.1. DNA extraction

After culturing the yeast on YPDA medium for 48-96 h, DNA extraction was carried out according to the method of Sampaio *et al*. (2001) . The cells collected on the surface of the petri dish were mixed with 500 µl of lysis buffer in a minicolumn, the equivalent of a volume of 150 µl of glass beads (0.25 to 0.30 mm in diameter) were added to 500 µl of phenol chloroform solution (1:1, v/v, pH 8.0). After 3 minutes vortexing, the suspension was then centrifuged for 30 min at 4°C to separate 4 phases: the glass beads (pellet), the phenolic phase (containing proteins and lipids), the cell debris and the aqueous phase (supernatant containing DNA and RNA). Approximately 400 µl of supernatant containing DNA and RNA was transferred to a new minicolumn to which an equal volume of ice-cold ethanol (96%) was added, homogenized and kept at -20°C for 30-60 min, allowing the precipitation of DNA. The recovered precipitated nucleic acids are dissolved in 100 µl sterile distilled water with 4 µl RNase to remove the RNAs. After incubation at 37°C for 30 min, 11 µl of sodium acetate (0.3 M) were added to 200 µl of ice-cold ethanol (96%). After final centrifugation, the DNA was washed with ethanol (70%), dried and resuspended. The extracted genomic DNA was stored at -20°C.

8.2.2. Amplification of DNA by polymerase chain reaction (PCR)

The amplification reaction (PCR) was carried out in a final volume of 50 µl containing 5 µl of Taq polymerase buffer (10X); 4 µl of $MgCl_2$ (25 mM); 10 µl of $dNTP_s$ (1.25 mM); 0.2 µl of Taq polymerase (5 U/µl; Sigma-Aldrich). 2.5 µl of each RLR3R (5′-GGTCCGTGTTTCAAGAC-3′) and V9G (5′-TGCGTTGATTACGTCCCTGC-3′) primer, 5 µl of genomic DNA and 20.8 µl of distilled water. The amplification reaction was performed in a thermocycler (Biometra). The amplification programme included an initial denaturation step of 5 min at 94°C, 30 PCR cycles (denaturation 45 seconds at 94°C, hybridization 30 seconds at 52°C, extension 2 min at 72°C) and a final extension cycle of 7 min at 72°C. The amplified DNA fragments were separated by electrophoresis on 1.4% agarose gels in TAE buffer (40 mM Tris-Acetate, 1.25 mM EDTA, pH 8.3) at 95 V for 2 min. On each gel, a molecular size marker was used as a reference. DNA band patterns were visualized under UV illumination using an imager. The presence of DNA was revealed by the fluorescence of the EtBr (Ethidium Bromide) trapped between the molecules (Turchetti *et al*. 2013).

8.2.3. Sequencing and phylogenetic analysis

Sequencing of the 600-650 bp region was performed using primer NL1 (5′-GCATCAATAAGCGGAGGAAAG-3′) and reverse primer NL4 (5′-GGTCCGTGTTCAAGACGG-3′), as described in the study of Turchetti *et al*. (2013). PCR products were sequenced using a commercial sequencing facility (Macrogen, Amsterdam, and The Netherlands). The public NCBI GenBank database was used to check for sequences compatible with the sequences obtained. A phylogenetic tree was constructed using the Neighbor-joining method with 1000 bootstraps (Saitou and Nei, 1987). Using the Kimura-2 parameter (Kimura, 1980) , phylogenetic distances were calculated in Molecular Evolutionary Genetics Analysis 11 (MEGA 11).

Part 2: Production and α-amylase optimization

1. Substrate preparation

Production of α-amylase is carried out using various agricultural wastes and industrial effluents as a basal substrate that were collected locally from oil mills such as olive pomace, mastic waste, leftover bread and potato peels. The wet substrates are dried, ground and sieved to obtain a 0.1 mm powder.

2. Inoculum preparation

After growth of the strain on 50 ml of YPGA medium in 250 ml Erlenmeyer flasks a volume of distilled sterile water is added, shaking is done manually to homogenize the cells. Cells were counted directly using a Thoma cell. 10^7 cells/ml from the culture were inoculated for alpha amylase production (Dakhmouche Djekrif *et al*., 2021).

3. Solid state fermentation (SSF)

Cultures were carried out in 250 ml Erlenmeyer flasks containing 10 ml of substrate moistened with 6 ml of distilled water. The medium was sterilized at 120°C for 20 min. Erlenmeyer flasks were inoculated with the previously prepared inoculum and then incubated at 30°C for 72 hours.

4. Submerged fermentation (SmF)

The olive pomace waste powder is diluted in distilled water at different concentrations (2%, 3%, 4%, 6% and 8%) to select the concentration that gives the best alpha amylase production. The mixture was heated and integrated under agitation until a homogeneous solution was obtained. It was then filtered through Whatman paper 1. The filtrate forms the base medium. Following sterilization, the Erlenmeyer flasks are inoculated with 10^7 cells/ml of *Geotrichum candidum*. The cultures were incubated under agitation at 30°C for 72 hours.

5. Enzyme extraction

Enzyme extraction from solid cultures is carried out as follows: a quantity of 5g of fermented substrate is vortexed with 25ml of phosphate buffer for 5min and then centrifuged at 4°C and 10000rpm for 10minutes. The obtained filtrate represents the crude extract (Sahoo, 2020).

6. Determining optimal growing conditions

6.1. Pre-optimization of α-amylase production by one factor at a time (OFAT) approach

The OFAT method was used to estimate the effects of the following conditions on α-amylase production by the yeast strain: temperature, pH, nitrogen and carbon sources.

Different fermentation temperatures from 30°C to 65°C were studied to get the optimum incubation temperature for the α-amylase, the effect of pH was studied at pH values 4.0, 5.0, 6.0. Buffer systems were used at a concentration of 0.1 M sodium citrate buffer to pH 4.0 and 5.0, sodium potassium phosphate buffer to pH 6.

To determine the effects of different carbon and nitrogen sources, the basic medium was supplemented with 1% of different organic and inorganic nitrogen source such peptone, malt extract, yeast extract, meat extract, corn steep, KNO_3, $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$. In addition, glucose, galactose, sucrose, fructose, maltose, lactose, soluble starch and potato starch have been added at 1% to the basal fermentation media. Cultures were incubated for a period of 72 hours.

6.2. Statistical experimental design for the screening and optimization of α-amylase production

6.2.1. Plackett-Burman design (PBD) for factors screening of α-amylase production

The Plackett–Burman design (PBD) was the statistical approach chosen for this optimization study. Initial screening of the most significant fermentation parameters was performed (Plackett and Burman, 1946); All the experiments are carried out according to a design matrix, which is based on the number of variables to be studied. The matrix applied to this study is constructed as follows: the 1st line corresponds to that of the generator chosen according to the number of factors studied (for example, for 7 factors we use the matrix N = 8), (Table 5) for the second line, the 1st sign must necessarily be the last sign of the 1st line and the following signs are those of the 1st line and continue (Table 6).

Table 5*:* Matrix generators for N= 8, 12, 16, and 20 Plackett and Burman design.

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

Eleven nutritional and physical factors including three dummy variables used to estimate the experimental error (soluble starch, malt extract, NH_4Cl, pH, inoculum, moisture content, $CaCl_2$, $MgSo_4$) were tested at two levels, high (+) and low (-) (Table 7) in the experiment based on the placket –Burman matrix design, alpha amylase activity was taken after 72h of fermentation and scored as U/g.

Table 6*:* Plackett–Burman matrix for the study of 11 factors with 12 experiments.

Experiment	**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

Note: factors:

A: Soluble starch , **B:** Malt extract , **C**: NH_4Cl, **D**: Dummy, **E**: pH, **F:** Moisture content, **G :**Inoculum, **H:** Dummy , **I**: $CaCl_2$, **J**: $MgSO_4$, **K**: Dummy.

- **+1** higher level**; -1** lower level.

6.2.2. Statistical analysis of the Plackett-Burman design

Statistical analysis was performed using Minitab 19 (Minitab, LLC, Pennsylvania, USA). The effect of each variable was determined by the following equation (Prajapati *et al*., 2014):

$$E(xi) = 2\left(\sum M_i^+ + M_i^-\right)$$

Xi: present one of the variable studied for example **A**

E(xi): is the effect of concentration of the variable tested, $\mathbf{M}_i^+$ **and** $\mathbf{M}_i^-$ showing α-amylase production in experiments where the variable (Xi) measured was presented at high and low concentrations, respectively.

The experimental error is defined as the mean of the error effects **(E_d)**:

$$V_{eff} = \sum (E_d)^2 / n$$

V_{eff} is the level effect variance, **(E_d)** is the level effect for the dummy variables and ***n*** is the experimental number of dummy variables.

The standard error **(SE, Es)** of the concentration effect was the square root of the effect variance.

$$SE = \sqrt{V_{eff}}$$

The significance of each variable is determined by Student's t test: Where ***E(xi)*** is the effect of the variable Xi.

$$t(Xi) = E(xi)/Es$$

If the t-test is significant, the impact is the result of a change in the level of the variable under study. This effect can be positive or negative.

Table 7: Minimum and maximum levels for the PBD factors studied.

Variables	Levels	
	-1	**+1**
Soluble starch g%	**0**	**1**
Malt extract g%	**0**	**1**
NH_4Cl g%	**0**	**0.5**
Dummy	**-**	**-**
pH	**4**	**8**
Moisture content % (v/w)	**50**	**80**
Inoculum (Cell/ml)	**10^5**	**10^7**
Dummy	**-**	**-**
$CaCl_2$ g%	**0**	**0.1**
$MgSo_4$ g%	**0**	**0.1**
Dummy	**-**	**-**

- **+1** higher level**; -1** lower level.

6.2.3. Central composite design (CCD) for optimization of α-amylase production

Based on the results of PBD, three significant parameters including malt extract, moisture content and $CaCl_2$ were investigated for their optimum interactions using RSM using central composite design at five different levels (Table 9) and it consists of three parts (Table 8); A 2^4 Factorial points (−1, +1), six axial points (−α, +α), and six replicates for central point (0) with a total of 20 experiments were carried out.

Table 8: Composite 3-factor central design structure.

Experiments	Variable Levels		
	A	**B**	**C**
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9	-1.682	0	0
10	+1.682	0	0
11	0	-1.682	0
12	0	+1.682	0
13	0	0	-1.682
14	0	0	+1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0

A : Malt extract , B : Moisture content , C : $CaCl_2$

- **A**: **X1** = (Malt extract – 1) /0.5
- **B: X2** = (Moisture content – 50) /15
- **C**: **X3** = ($CaCl_2$ – 1) /0.5

X 1, X 2, X 3, are the derivative of equation solutions obtained after processing the CCD matrix.

They allow us to determine the positions of the extremums of these functions.

$$\mathbf{X} = ([\mathbf{Value\ Concentration\ of\ the\ variable\ under\ study}\ ] - \mathbf{Y})/\mathbf{Z}$$

1. **Y**: is the value at level 0 of the factor studied.
2. **Z**: is the step chosen by the manipulator.

Table 9: Relationship between the levels coded and the real levels of the factors used in the CCD.

Variables	**Levels**				
	-α	-1	0	+1	+α
Malt extract g%	0.159	0.5	1	1.5	1.841
Moisture content %	24.77	35	50	65	75.23
CaCl2 g%	0.159	0.5	1	1.5	1.841

7. Analysis and modeling

To study the impact of the variables and modeling the response as an equation, a matrix integration is performed using Minitab v19 software. Statistical analysis of the Y response is determined either by looking for optimums/minimums, or by fixing one of the parameters. The graphical representation allows rapid visualization of the response as a function of the studied parameters. The response data underwent an analysis of variance (ANOVA) to obtain key metrics such as the F-value (indicating the overall model significance), P-value, lack of fit F-value, and R^2 value (coefficient of determination, assessing the goodness of fit for the regression model).

8. Validation of the experimental model

The α-amylase production was carried out in 250 mL conical flasks consisted olive pomace medium which was optimized according to well-defined parameters by one factor per time, Placket-Burman design and response surface methodology with central composite design of Box-Wilson optimization approaches to confirm and validate the predicted value and experimental value of enzyme production.

9. Kinetics for α-amylase production

The evolution of amylase produced by the yeast *G. candidum* PO27 during the different growth phases was carried out in batch using 250 ml Erlen flasks on based pomace olive optimized medium. Incubation is carried out at 60°C in order to study the kinetics of enzyme production as well as the evolution of the quantity of proteins and carbohydrates and the variation in pH for a period of 100h. Samples are taken periodically every 4 hours, and then centrifuged at 10,000 rpm for 15 minutes at 4°C. The various assays (α-amylase activity, proteins, and sugars) are determined from the supernatant. The experiments were carried out in duplicate.

10. Analytical methods

10.1. α-Amylase assay

The DNSA (3,5-dinitrosalicylic acid) method is commonly used for the estimation of reducing sugars. This method is based on the interaction of reducing sugars with the DNS reagent, which is yellow in its oxidized form, in alkaline and warm environments resulting in the formation of a red-colored compound (Figure 6) The intensity of this red color is directly proportional to the concentration of reducing sugars, which can be measured by spectrophotometry at a specific wavelength.

Figure 6: Determination of reducing sugars by DNSA.

α-Amylase activity is determined by the starch hydrolysis capacity of the crude enzyme extract (Annex 3). The amylase activity assay is determined as described by (Bernfeld, 1955):

1. Add 0.5ml of crude enzyme extract to 0.5 ml of 1% starch in buffer (phosphate pH 5).
2. Incubate the reaction mixture at 40°C for 30 min and cool immediately.
3. Stop the reaction by adding 1 ml of DNSA solution and incubate at 100°C for 10 minutes.
4. Read the absorbance of the samples at 540 nm.

The reaction is colorimetric and the intensity of the coloration is proportional to the amount of reducing sugar released by the hydrolysis reaction. The α-amylase activity is defined as the amount of enzyme extract required to produce 1 µmole of reducing sugar (equivalent to maltose) per min.

The quantity of reducing sugars is determined on a calibration curve (Annex 3) with maltose at concentrations from 0-2 mg/ml.

10.2. Dry weight determination

Dry weight of the samples was determined by drying them in a hot air oven at 70°C for 72h. The supernatant represents the crude enzyme extract is used to assay enzyme activity (proteins and sugars in the case of the kinetic study).

10.3. pH measurement

The pH of the enzyme extract is measured after the enzyme extraction (supernatant) using a pH meter (GLP 21, Spain), previously calibrated.

10.4. Sugar Carbohydrate determination

Using the Dubois method, concentrated sulfuric acid causes dehydration of the oses under heat, with the formation of hydroxy-methyl furfural (in the hexose case) and furfural (in the Pentose case). These compounds condense with phenol to give colored compounds; the intensity of the color is proportional to the concentration of oses (Dubois *et al*. 1956) (Annex 3).

10.5. Determination of protein content

Protein estimation was done by the Bradford method of using bovine serum albumin (BSA) as standard. Reaction mixture contains 3.0 ml of Bradford reagent and 0.1 ml of the extract. The incubation was at room temperature for 5 min followed by recording absorbance at 595 nm (Bradford, 1976).

Part 3: Purification and characterizations

1. Preparation of the crude extract

After 40 h incubation at 60°C of the yeast *G. candidum* on optimized medium. The extraction of the enzyme from the culture was performed by centrifugation. The supernatant constitutes the crude enzyme extract.

2. Concentration and Ultrafiltration

Alpha amylase from *G. candidum* PO27 was purified from a 40ml crude extract of an optimized solid medium. The crude extract was concentrated by ultrafiltration on Amicon stirred cell (Slater *et al*., 1986) and Amicon tube with a cut-off of 10 kDa and 5Kda.

Figure 7: Amicon stirred cell concentration.

3. Purification by molecular exclusion chromatography

Using the sample loop, 2 ml of the concentrated extract was processed at a flow rate of 1 mL/min on a Superdex 75 column, previously equilibrated with 20mM pH7 Tris HCl buffer. Chromatographic separations were performed on an AEKTA start. Proteins retained on the column were eluted with the same buffer. Amylase activity was monitored at all stages. The Bradford method using Bio-Rad dye reagent determined protein concentration. A standard curve was generated using bovine serum albumin (BSA).

4. SDS PAGE electrophoresis

The SDS page (Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis under denaturing conditions) is used to determine the number of protein subunits and their molar mass. The sample is fractionated by 14% polyacrylamide gel electrophoresis and are separated according to their molecular weight, determined using protein ladder (Pierce unstained protein MW marker). The procedure and composition of the various electrophoresis solutions and gels are detailed in (Annex 4).

5. Determination of the total carbohydrate of the pure enzyme

The rate of glycosylation or the total carbohydrate content of the pure enzyme was determined after hydrolysis by the Dubois *et al*'s method (1956) .The method is described in Annex 3. Color density is then measured at 488 nm against a standard range of 100 µg/ml maximum mannose. The amount of carbohydrates is expressed as the equivalent amount of mannose (Ichikawa *et al*., 2014) .

6. Characterizations of purified enzyme

6.1. Temperature- and pH-dependent activity

The influence of temperature on the activity of the amylase enzyme was studied at pH 5 (0.1 M citrate phosphate buffer). Measurements were taken over a range from 30°C to 100°C to determine the optimum enzyme temperature.

The variation in enzyme activity as a function of pH was studied at the optimum temperature for each enzyme over a pH range of optimum temperature for a pH range of 3.0 to 11.0.

Three types of buffer solutions were used to study the effects of pH on enzyme activities: 0.1M citrate buffer for pH range 3.0 to 5.0, 0.1M, 0.1M phosphate buffer for pH range 6.0 to 8.0, and 0.2M glycine-NaOH buffer for pH 9.0 and 11.0.

6.2. Study of thermal stability

The enzyme's thermostability was tested by incubating the pure enzyme extract in citrate buffer (0.1M, pH 5) at two temperatures (70°C and 100°C) for up to 3 hours, the enzyme solution is distributed in equal volumes in separate tubes, which are heated together in a water bath at a carefully adjusted temperature. The different samples are taken out one by one at set times (every 30 minutes) and cooled instantly in an ice bath. After each heat treatment, the α-amylase activities were measured.

6.3. Determination of the kinetic parameters

Different substrate concentrations ranging from 1 to 5% soluble starch were tested to determine the kinetic parameters (V_{max} and K_m) of α-amylase. Measurements were carried out at the optimum temperature and pH of the enzyme using the inverse coordinate representation mode of Lineweaver and Burk (1934).

6.4. Effect of differents metals and chemical reagents

To study the effect of ions on α-amylase activity, salts such as NaCl, KCl, $FeCl_3$, $ZnCl_2$, $HgCl_2$, $CuSO_4$, $MgSO_4$, $CaCl_2$, $NiSO_4$ and $MnSO_4$. Amylase samples are incubated with each metal ion (5mM) separately at 60°C for 30 min in 0.1M citrate phosphate buffer, pH 5 and then α-amylase activities are measured (Djekrif-Dakhmouche, 2016) .

Other chemicals such as EDTA, urea, SDS, β-mercaptoethanol, Tween 80, Tween 20, Triton X-100, ethanol, DMSO and acetone were tested on enzyme activities with a concentration of 1%, w/v.

The residual α-amylase activity of the samples is calculated relative to the control (in the absence of an effector).

7. Application of α-amylase as a desizing agent

To study the desizing efficiency of α-amylase isolated from *Geotrichum candidum* PO27, as described by Nair and Bhat (2020), white tissue (5 cm x 5 cm) was starch-treated with a 5% starch solution and dried. The starched tissue strips were then immersed in a solution of potassium iodide (0.1; 1%). Strips of starched tissue were then immersed in flasks containing (a) Untreated (control) and (b) 25 ml tap water (control) (c) 24 ml tap water and 1 ml purified *G. candidum* PO27 α- amylase (d) 24 ml tap water and 1 ml commercial *Aspergillus oryzae* α-amylase. Finally, the effectiveness of starch removal was observed and evaluated by photographing the stained cloth strips.

8. Statistical analysis

The results were processed using Minitab 19, a software program designed for data analysis. All experiments were performed in duplicate, data were analyzed using a One-Way ANOVA followed by a Turkey multiple comparison test, with a chosen level of statistical significance of 0.05.

Results and discussion

Results and discussion

In order to study the production of a novel yeast enzyme for use in industrial biotechnology, We will start by presenting the outcomes of isolating and identifying amylolytic yeasts from various sources in eastern Algeria. Subsequently, we will choose the most effective yeast strain for α-amylase production. In the second part, we will showcase the results of enzyme production, its optimization, and a kinetic study. Furthermore, we will disclose the results of purifying the studied enzyme, along with examination its biochemical characteristics and associated kinetic parameters, and the effect of different metal ions and chemical reagents, finishing with a trial application of the enzyme and validation of its efficacy in industrial fields.

1. Isolation

In this study, a total of six samples were collected from several regions to obtain yeasts capable of producing α-amylase, cellulase, esterase and lipolytic enzymes. After isolation, the pH results are taken on the basis of the pH measurement of the samples' stock solution using a pH meter.

It is found that the four samples; olive pomace and its rinse water, mastic oil cake and vegetable smen have an acidic pH of 4.4, 6.30, 6.20, and 6.33 respectively. The pH of the thermal water is neutral at 6.98, while the pH value of the olive forest soil is basic at 7.39. Generally, yeasts prefer a slightly acidic environment and their optimal pH is between 4.5 and 5.5. Nevertheless, they grow at a wide range of pH and adapt to pH 3-10. In addition, several species can grow at strongly low pH values as low as 1.5 (Péter and Rosa, 2006) . Generally, habitats with basic pH values are quite difficult for yeast life, while some species can grow successfully at pH 10 or above (Buzzini *et al*., 2018).

From one hundred yeast strains isolated. 78 yeast strains were selected for their rapid growth on YPDA (Figure 8). Based on higher incidence, 27 yeast strains were selected from the olive pomace, of isolated yeasts and six strains were isolated from olive rinse water (RW). Misbah *et al*' research, (2019) proved olive rinse water (OR), olive pomace (OP) and olive mill wastewater (OM) are a favorable media for the development of enzymes producing microorganisms. Therefore, 16 strains were isolated from soil (OS) with similarity in colony morphology in most isolates, which agrees with the results of Williams *et al*. (2021) who isolated 14 yeast obtained from palm oil impacted soil.

Twelve strains were selected from mastic oil cake (MC). To our knowledge, this source, used for the isolation of α-amylase, cellulase and lipase producing yeasts, has not been discussed before, despite their richness, as previous study has demonstrated the importance of oily cakes as a source for the isolation of microorganisms for biotechnological use (Singh *et al*., 2014).

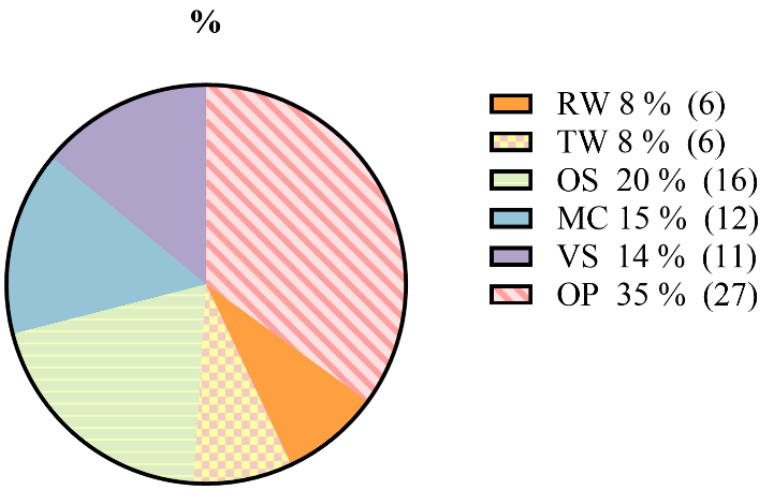

Figure 8: Frequency of isolated yeast strains.

Springs hot water is one of the major sources of thermostable enzymes produced by yeasts. The isolation of yeast from thermal water (TW) was unsuccessful using the successive dilution technique while using an enrichment method made it possible to obtain 6 heat-resistant strains. Khadka *et al*. (2022) succeeded in isolating with the enrichment method about 44 of the bacterial strains from Kharpani hot spring water including *Geobacillus* sp. KP43 gave a high cellulase production. Also, *Geobacillus* (K1C) bacteria, was isolated from Manikaran hot springs and also selected for its high thermostable α-amylase production (Sudan *et al*., 2018). Eleven strains of vegetable smen origin (VS) were obtained, and this traditional butter has been the subject of many scientific studies. However, no detailed study has ever characterized its microbial diversity, including yeasts indeed. In the studies cited above, the microbial diversity focused mainly on the diversity of proteolytic lactic acid bacteria and, in a few cases, yeasts in traditional Algerian butter and on yeast diversity in different types of cheeses (Karam and Idoui, 2008).

2. Screening of enzymes producing yeasts

Hydrolysis starch by α-amylase produced a light purple area around the colony after revelation with Lugol solution. The absence of a clear zone indicates a reaction between iodine reagents and non-hydrolyzed starches in the starch Agar medium (Kwon *et al.*, 2020).The iodine-starch reaction is due to the formation of helical amylose and iodine as I_3- that fills the helical nucleus (Gunam *et al*., 2021). Halo zone formation and amylolytic index (AI) were used as a semi-quantitative method to classify isolates as being highly amylase producers (AI>1.5) (Muriithi *et al.,* 2021). Twenty-seven yeast strains were able to hydrolyze starch among these strains, RW1-3, RW1-2 and PO27, showed high amylolytic activity (Figure 9).

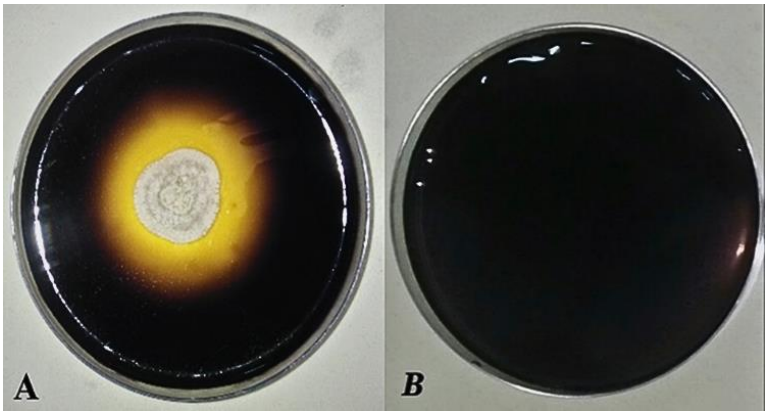

Figure 9: Amylase qualitative assay for the selected isolate PO27 (A: Presence of selected yeast, B: Absence of selected yeast).

Olive oil mixed with agar medium represents a good choice for selecting of lipase positive strains (Lanka and Latha, 2015) with an indicator like red phenol (Bharathi and Rajalakshmi, 2019). After incubation on olive oil medium, the dishes show yellow areas around the wells due to free fatty acids released by the lipolytic organisms, which lowers the pH of the medium from phenol red to yellow, indicating the presence of lipase (Lanka and Latha, 2015). Twenty-five strains demonstrated lipolytic activity. However, the PO27 was the most efficient because it has shown the highest diameter on olive oil (Figure 10). All other strains exhibited low and moderate lipolytic activity. The clear zone around the colony showed that the isolates were also capable of promoting cellulase hydrolysis (Arman *et al.*, 2020). Among the 78 yeast strains, most had no cellulase activity except five strains showed weak activity including the yeast PO27, PO6, PO8, PO20 and RW2-2 (Figure 11). Also, tween 80 is used as a substrate for esterase or lipase screening, no color is required for visualization, they give the opaque zones around the colonies which are the precipitation zones of the calcium salt, while hydrolysis

indicates either esterase or lipase activity (Wadia and Jain, 2017)(Figure 12). Seventeen strains showed esterase activity, and all species exhibited moderate and high esterase activity, including the amylolytic the cellulolytic and the lipolytic yeast isolate PO27 (Figure 13).

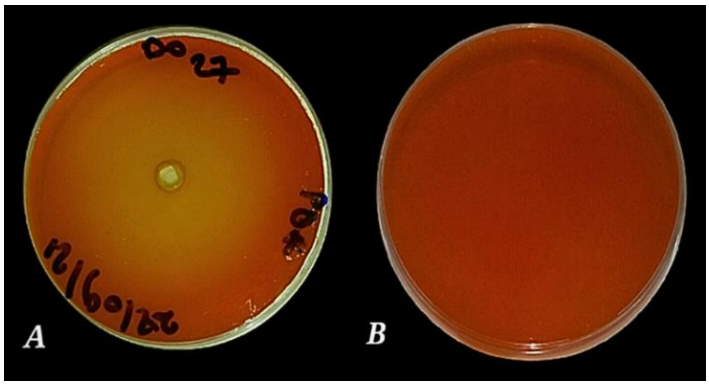

Figure 10: Lipase qualitative assay for the selected isolate PO27 (A: Presence of olive oil, B: Absence of olive oil).

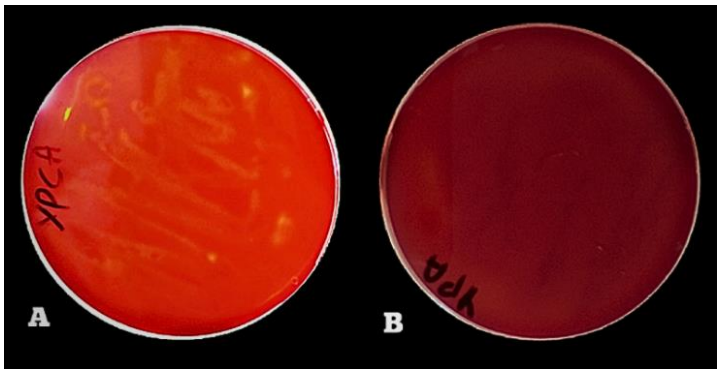

Figure 11: Cellulase qualitative assay for the selected isolate PO27 (A: Presence of carboxymethyl cellulose , B: Absence of carboxymethyl cellulose).

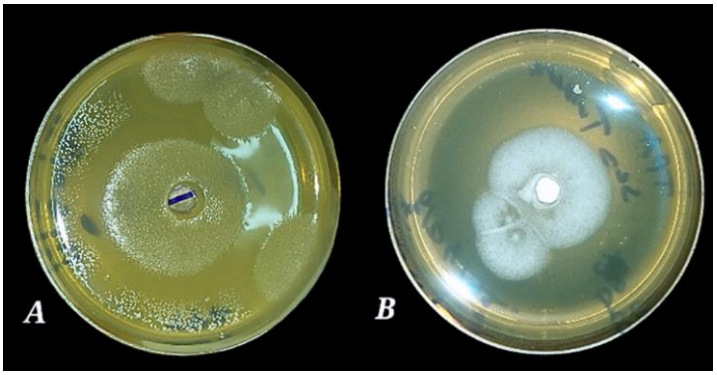

Figure 12: Esterase qualitative assay for the selected isolate PO27 (A: Presence of tween 80, B: Absence of tween 80).

In addition, yeast biodiversity varied between the six isolation samples (Figure 13). The results of the experiment revealed that most of them present a good biotechnological interest, due to their capacity to tolerate high yeast concentrations. However, the maximum number of yeast colonies obtained from the olive pomace sample indicates that the majority of them have a more diversified and important enzymatic potential.

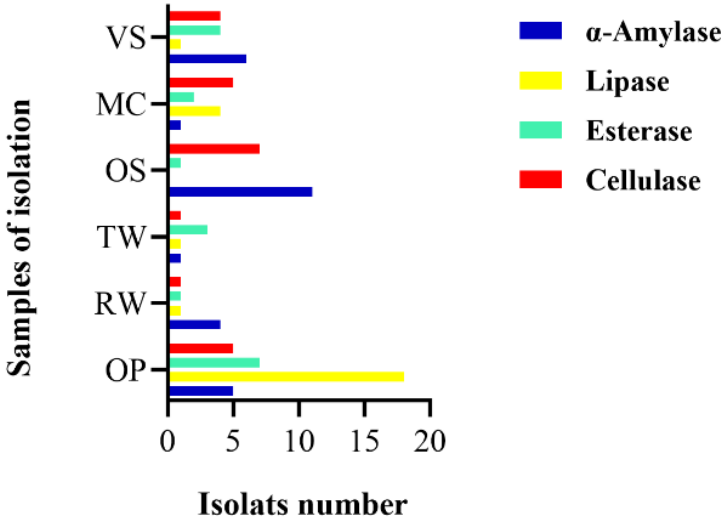

Figure 13: Distribution of enzymatic activities on the six samples. Bars: amylolytic isolated strains (**blue bar**)، lipolytic isolated strains **(yellow bar)**، esterolytic isolated strains (**green bar**)، cellulolytic isolated strains (**red bar**).

As a result, the yeast PO27 olive pomace origin is selected for its high capacity to produce α-amylase and its enzymatic diversity profile. The PO27 isolate showed a 40 mm α-amylase halo and a 1.6 of AI, a 38mm of lipase halo and 22 mm opaque halo in tween substrate with 1.1 of enzymatic index. It also showed a positive result with the cellulase qualitative test, and which is not interesting for industrial use because of its low activity. Following the screening and identification of the isolate, it was found that our results are consistent with those of previous studies (Zaier *et al.*, 2021), in which their isolated *Geotrichum candidum*, showed an ability to hydrolyze and assimilate several carbon sources including starch, tween, casein, and CMC through their production of enzymes such as amylase, lipases (or esterase), protease and cellulase. Furthermore, there are many reports in the literature about the production of lipases from *Geotrichum candidum* (Hlavsová *et al.*, 2009). Different strains have shown the enzymatic potential of this yeast species including *Geotrichum candidum* CMSS06 which produced α-amylase (Attanayaka *et al.*, 2009), *Geotrichum candidum* Strain Gad1 able to

produce cellulase (Gad *et al.*, 2022), *Geotrichum candidum* 3C produced endo-1,4-xylanase (Rodionova *et al.*, 2000), *Geotrichum candidum* AA15 also exhibited the capacity to produce pectinase (Ahmed *et al*., 2020) and *Geotrichum candidum* QAUGC01 allowed the production of serine alkaline protease (Muhammad *et al.*, 2019).

3. Identification and characterization of the selected yeast strain

The species presents a yeast with a fuzzy, filamentous appearance, with a mold-like aspect, in addition to its rapid growth; the colonies are white and have a velvety white cottony mycelium as described by Attanayaka *et al.* (2009). Under the light microscope, its shape is rectangular and then rounded, and showed many arthrospores (arthroconidia) which are consistent with the previous study (Loo, 2006) that clearly shows conidia (arthrospores) with variable width. Based on morphological characterization, this yeast species was identified as *Geotrichum* sp. (Figure 14). The species has been confirmed by molecular identification.

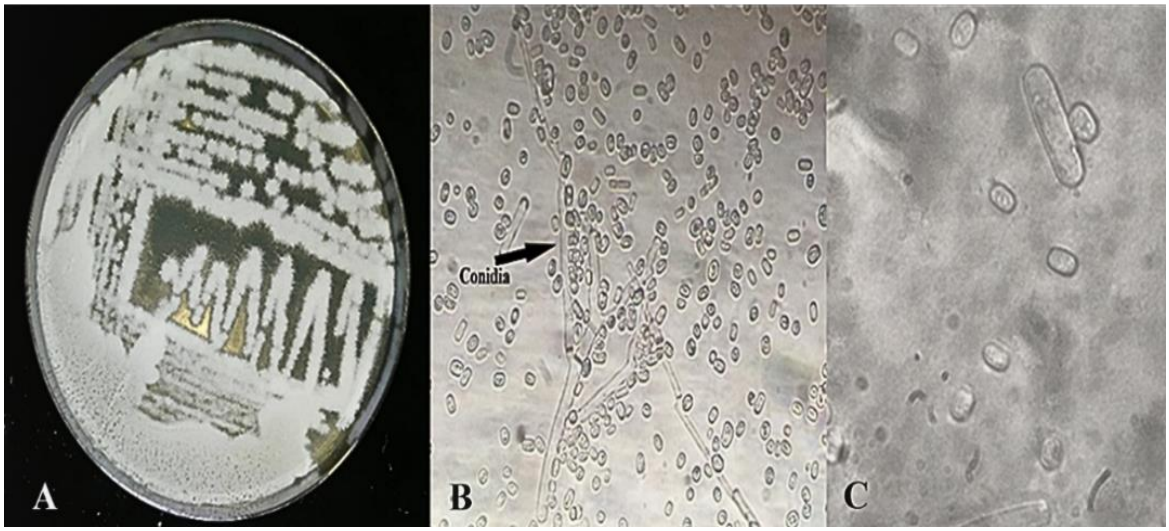

Figure 14: Morphology observation of PO27 strain cultured on YPD agar plate for 48 h at 30 °C (A: Macroscopic observation, B: Microscopic observation G:40 X, C: Microscopic observation G:100 X).

After molecular identification from sequencing of the D1/D2 domain of the 26S gene sequence, the length of the D1/D2 region was found to be 699 bp (Annex 2). Sequence comparison of PO27 isolate (GenBank accession no. PP024529) with those included in the GenBank database, shows 99.57% similarity with *Geotrichum candidum* strain CV2 (GenBank Accession no. KX364934) and illustrated a high similarity to its teleomorph *Galactomyces candidum.* According to the phylogenetic analysis (Figure 15), the isolate PO27 cluster in a branch near to *G. candidum* with more than 77% probability, which is well supported. Based

on its morphological and molecular characteristics we identified the isolate PO27 as *Geotrichum candidum* and not as its teleomorph *Galactomyces candidum*, since no ascospores were observed.

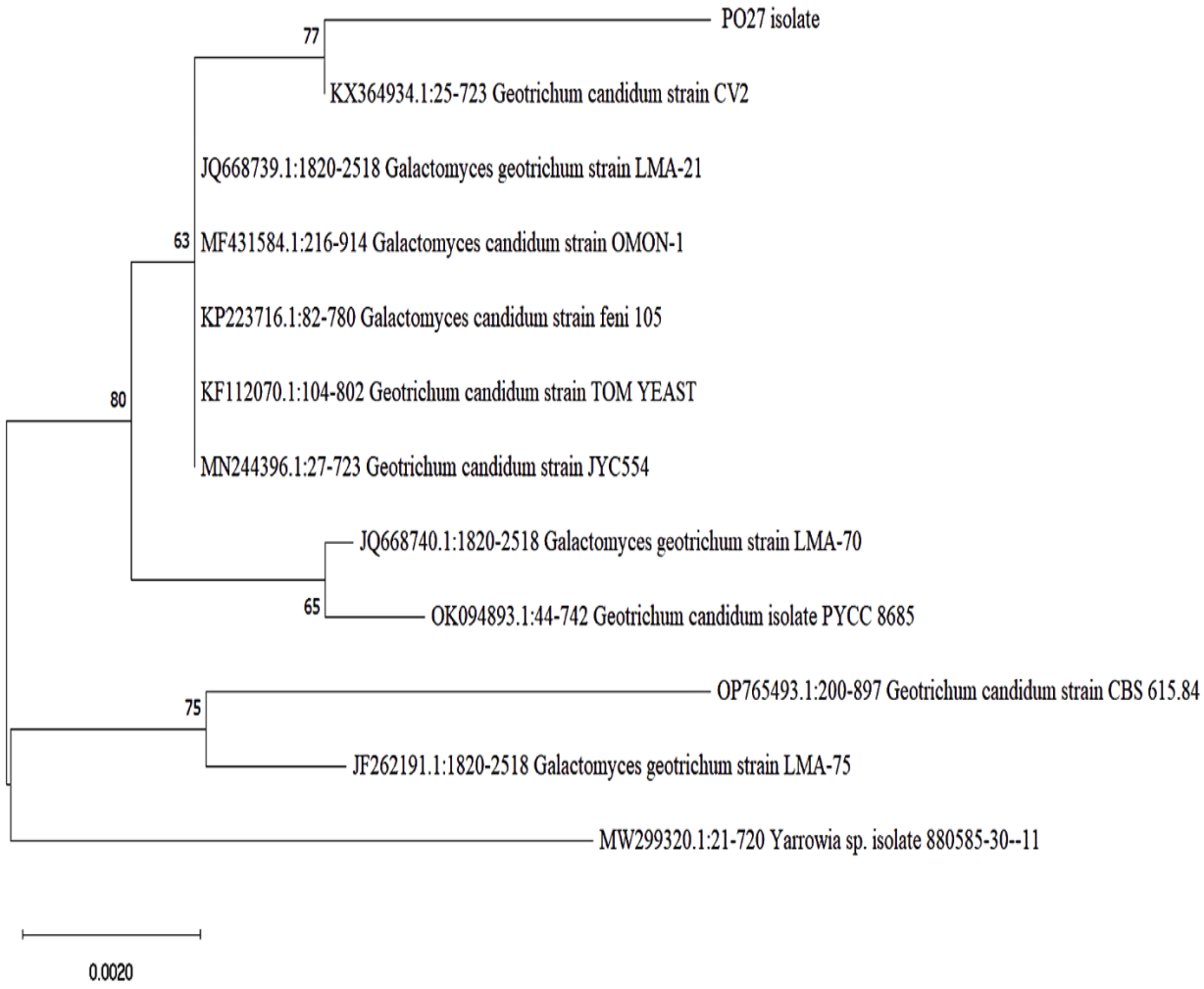

Figure 15*:* Phylogenetic relationships of PO27 and other closely related *Geotrichum* sp. using NJ method with bootstrap value of 1000 replicates. *Yarrowia* sp. was used as outgroup. Bootstrap values (>50 %) were shown at the nodes. Scale bar, 0.002 nucleotide substitution rate units.

4. Production of α-amylase studies

In solid-state fermentation (SSF), the most important factor is the choice of a suitable medium for the production of an enzyme. In this study, the selected strain *G. candidum* PO27 was inoculated into solid residues such as potato peels, bread leftovers, olive pomace, and mastic oil cake. The produced α-amylase was then quantified via the DNS method. Among the four agro-substrates tested, a significant difference (p=0.038) was obtained with olive pomace 180.71 U/g to be the best solid substrate for the α-amylase production followed by potato peels 150.63 U/g, mastic oil cake 132.66 U/g and bread leftovers 109.04 U/g (Figure 16). This can be explained by the differences in the texture and chemical composition of the substrates.

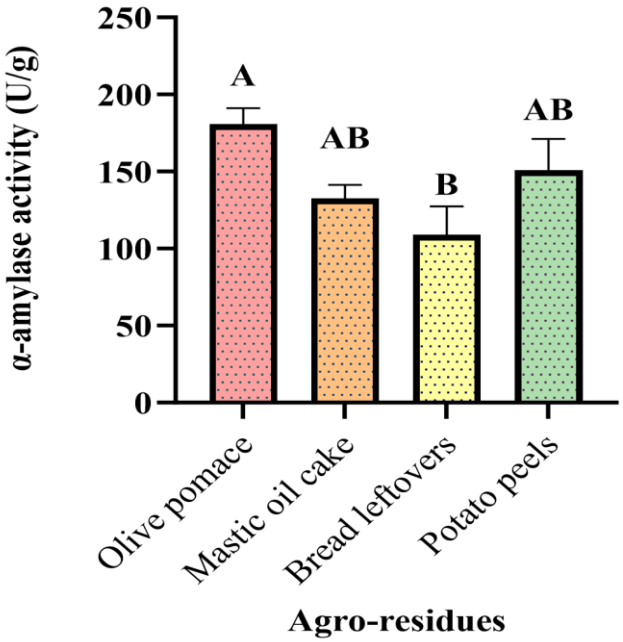

Figure 16:α-amylase production by *G. candidum* PO27 under solid-state fermentation (Tukey method: Means that do not share a letter are significantly different).

The amylases production from agro-industrial waste is intended to resolve pollution problems and to obtain a low-cost medium. Literature reports suggest that *Aspergillus awamori* isolated from olive cake, gave the highest amylase activity of 230 U/g using olive oil cake (Karam *et al.*, 2017). In contrast to Obi *et al.* (2019), potato peel was obtained as the best substrate with the highest amylase production (2.36 U/ml) from *Bacillus subtilis*. Using various oil cakes as low-cost substrates, such as groundnut oil cake (GOC), coconut oil cake (COC), and sesame oil cake (SOC) *by Aspergillus oryzae*, Balakrishnan *et al.* (2021) also studied α-

amylase production under SSF. Their results showed that oil cake (GOC) was the best substrate for maximum α-amylase production of 9868.12 U/g, followed by 4031.12 U/g using COC and 3068.15 U/g using SOC.

In another study, using *Bacillus subtilis* (Khelil *et al.*, 2022) to produce α-amylase, it was shown that stale bread had the highest amylolytic activity (107.3 U/min), whereas potato peel yielded 55.5 U/min. Furthermore, Benabda *et al.* (2019) demonstrated that SSF from *Rhizopus oryzae* produced α-amylase (100 U/g) using bread waste as a substrate, which is very similar to our solid-state fermentation results on leftover bread. Agricultural substrates such as wheat bran, rice bran, maize bran, corn bran and wheat straw have attracted attention for amylase production (Pranay *et al.*, 2019). Moreover, the capacity of several fruit peels, including banana, orange, and pineapple peels, to provide alternative carbon sources for α-amylase production was examined (Djekrif-Dakhmouche *et al.*, 2006 and Almanaa *et al.*, 2020) . Additionally, Singh *et al.* (2022) found that solid-state fermentation of apple peel could yield 17468 U/l of α-amylase from the *Bacillus subtilis* BS1934 strain. In another study, watermelon rinds (WMR) were used to produce α-amylase from *Trichoderma virens* (Abdel-Mageed *et al*., 2022).

In order to choose the best conditions for enzymatic production, submerged fermentation is carried out at different olive pomace substrate concentrations from 2% to 8%. The analysis of the experimental results by the ANOVA reveals that the difference in activity as a function of substrate concentration is highly significant (p=0.000), shows that 6% is the best with 34.395 U/ml, compared to other concentrations, followed by 4% with 32.8 U/ml. In comparison, 2% and 8% exhibited low activity with 30.115 U/ml and 24.490 U/ml respectively (Figure 17).

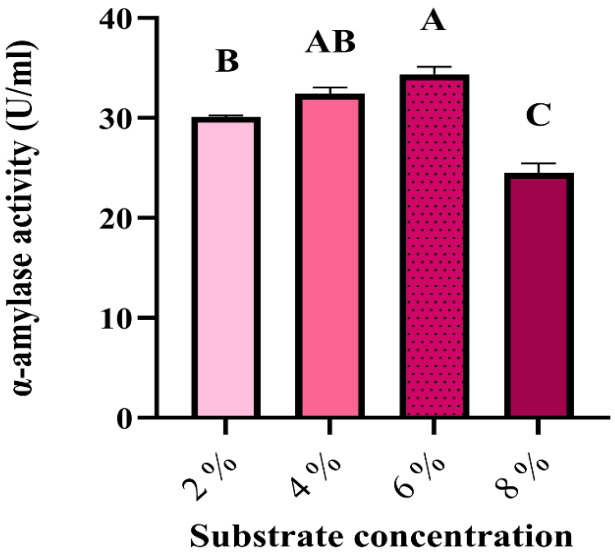

Figure 17: α-amylase production by *G. candidum* PO27 under submerged fermentation (Tukey method: Means that do not share a letter are significantly different).

Comparing our results is challenging because this is the first study to use *G. candidum* as a source of α-amylase by submerged fermentation using olive pomace. In addition, it is necessary to consider that our study is very attractive because of the dispensation of any mineral supplementation that makes it a suitable low-cost environment for α-amylase production by *G. candidum* PO27 (34.395 U/ml). Using a synthetic media, the highest production of the species *Geotrichum candidum* CMSS06 reached 6.4 U/ml of α-amylase at 72h. whereas *Aspergillus* spp. showed a maximum activity of 1.2335 U/ml at 96h (Attanayaka *et al.*, 2009) .

Compared to Divya and Padma (2014), which used a synthetic YEPD broth supplemented with starch, the amylase activity was 130 U/ml by the isolate *Geotrichum* sp. In contrast to Falih (1998), using Czapek-Dox medium, soil yeast *Geotrichum candidum* was found to have an amylase activity of 50µg/ml while the maximum amylolytic activity of *Geotrichum capitatum* was 34µg/ml. Moreover, the highest growth rate of *Saccharomyces cerevisiae* has been observed in the treatment with 2% potato peels in submerged fermentation, which increases amylase activity (Najmalddin *et al.*, 2023). Because of their low cost, availability and simplicity, oil cakes like cocos nut oil cake were used as a carbon source in liquid fermentation to produce α-amylase by *Aspergillus flavus*, which showed higher enzyme activity (170.3µg/ml) (Arunsasi *et al.*, 2010).

In recent years, the technique of the SSF process has been developed and used more extensively because of its simplicity, low cost, the simple need for fermentation equipment, better productivity, and less water production (Olakusehin and Oyedeji, 2022). As shown in Figure 18, α-amylase activity in solid-state fermentation (45.47U/ml) was highly significant ($p=0.038$) when compared with the activity in submerged fermentation (34.395U/ml). This result corroborates that of Jesubunmi and Ogbonna (2022), who reported the production of glucoamylase and cellulase by both *Fusarium* sp. and *Rhizopus* sp. was significantly higher in solid state culture ($p<0.05$) than in suspended culture. But that doesn't prevent about 90% of industrially important enzymes have traditionally been produced by submerged fermentation because of ease of handling and sterilization and better control of environmental factors such as temperature and pH (Oussadi and Kitouni, 2015). Furthermore, Premalatha *et al.* (2022) reported that the production of extracellular α-amylase from *Aspergillus tamarii* using wheat bran (WB) performed better in the SSF method than with SmF and achieved a higher α-amylase activity (519.40 U/g).

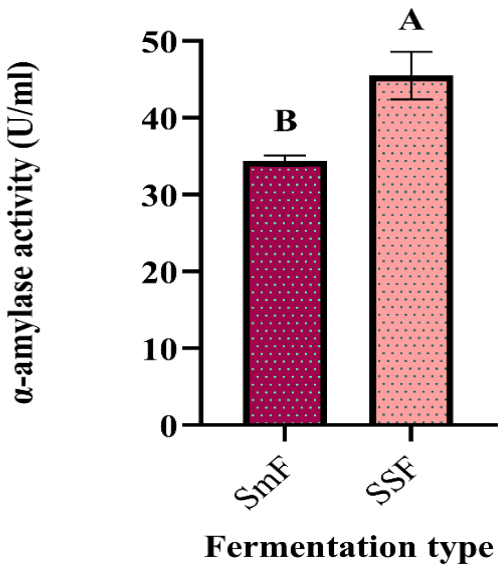

Figure 18: Comparison of α-amylase production by *G. candidum* PO27 in solid state and submerged fermentation (Tukey method: Means that do not share a letter are significantly different).

5. Pre-optimization of α-amylase production by one factor at a time (OFAT) approach

5.1. Temperature effect on α-amylase production

The amylolytic strain seems to be thermophilic. Maximum activity was obtained (191.10 U/g) at 60°C (p=0.000) followed by 186.35 U/g at 55°C (Figure 19.a). Enzyme production drops at a temperature of 65°C and gives a 121.68 U /g indicating the inhibition of amylase production, probably by suppression of cell viability and enzyme inactivation (Ahmed *et al.,* (2019). These results agree with several studies. Luang-In *et al.* (2019) showed that the optimal temperature for amylase of *Bacillus* sp. 3.5AL2 was 60°C and Finore *et al.* (2014) obtained maximum α-amylase secretion from *Anoxybacillus amylolyticus* at 60°C. The results showed *G. candidum* PO27 α-amylase is thermophilic enzyme with potential use in industrial processes. Microorganisms able to grow optimally at temperatures between 50°C and 60°C are known as moderate thermophiles. It can be assumed that moderate thermophiles, which are closely related phylogenetically to mesophilic organisms, can adapt to life in warm environments (Djekrif-Dakhmouche, 2016).

5.2. pH effect on α-amylase production

An optimal pH is an essential factor for the stability of the enzyme produced. Enzymes are pH sensitive and therefore production process pH must be carefully controlled (Mathew *et al.,* 2016) . The extracellular α-amylase activity was significantly higher (p=0.002) in pH 4 (178,61U/g) followed by pH 5 (158,145U/g), but the enzymatic activity was considerably lower in pH 6 (115,075 U/g) which indicates acidophilic nature of the isolated *G. candidum* PO27 (Figure 19.b). Similar to Fossi *et al.* (2005) who found that pH 4.5 was optimum for amylase production at 30°C by an isolated yeast. Also Olakusehin and Oyedeji, (2022) revealed that the optimum pH for α-amylase production by *Aspergillus flavus* S2-OY was found to be 5. On top of that, the pH measurement of the optimized medium of the *G. candidum* CMSS06 strain proves that the strain appropriates the acidic medium as the pH of the culture supernatant has dropped from 4 to 3.46 (Hlavsová *et al.,* 2009) .

5.3. Carbon source effect on α-amylase production

The carbon source is one of the major factors affecting enzyme production, particularly when it performs an inducer role. The α-amylase production by *G. candidum* PO27 was significantly (p=0.000) higher in the presence of soluble starch (151, 5 4 U/g) followed by galactose (148,315 U/g) but the enzymatic activity was considerably lower in addition of the other carbon source compared to the SSF without carbon supplementation (Figure 19.c). This

can be explained by the high carbohydrate content of olive pomace (Skaltsounis *et al.*, 2015), effective for the α- amylase production. This enzyme is extracellular and its production is induced by its substrate at a certain limit concentration (Djekrif-Dakhmouche *et al.*, 2006) . In addition to its role as an inducer, starch stabilizes the enzyme (De Mot and Verachtert, 1986) . Similar to Almanaa *et al.* (2020) who produced maximum amylase from *Bacillus subtilus* D19 using starch as carbon source. Also the α-amylase production from *Streptomyces* sp. Al-Dhabi-46 was found to be maximum in the culture medium containing 1% starch as carbon source (208 ± 11.4 U/ml) (Al-Dhabi *et al.*, 2020). In addition, galactose had a positive impact as the highest enzymatic performance (944 U/gds) by *Penicillium chrysogenum* in solid state fermentation (Ertan *et al.*, 2006).

5.4. Nitrogen source effect on α-amylase production

Due to the low nitrogen content of olive pomace, α-amylase production was enhanced by the addition of various nitrogenous compounds. From the result of the figure 19, it appears that *G. candidum* PO27 can use malt extract to enhance significantly ($p=0.000$) α-amylase production and achieve maximum production (270, 52 U/g). With few investigating the use of malt extract as a nitrogen source to improve amylase production, maximum α-amylase production using malt extract by *Pseudomonas balearica* VITPS19 has been reported by Kizhakedathil and Subathra Devi (2021). Although the inorganic source, NH_4Cl gave best α-amylase production (199.18 U/g) compared with the other inorganic sources used. Ahmed *et al.* (2017) revealed its insignificant effect on bacterial α-amylase production.

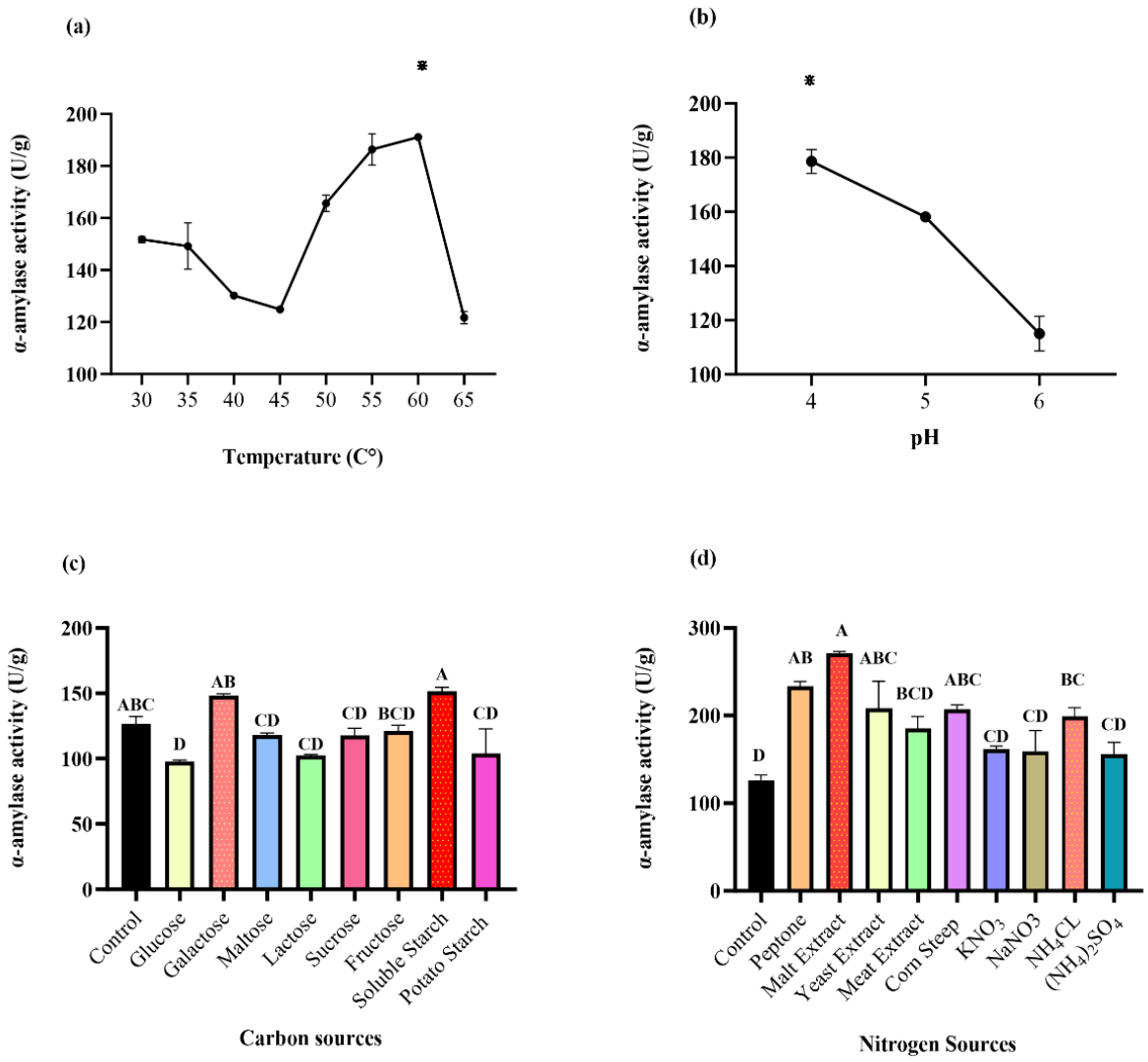

Figure 19: Optimization of α-amylase production by *Geotrichum candidum* PO27 by OFAT approach. Effect of (a) Temperature, (b) pH, (c) Carbon sources, (d) Nitrogen sources on α-amylase production, (*) indicate significant difference, (Tukey method: Means that do not share a letter are significantly different). $p < 0.05$).

6. Statistical experimental design for the screening and optimization of α-amylase production

6.1. Plackett-Burman design (PBD) for factors screening of α-amylase production

In this study, optimization of α-amylase production by *Geotrichum candidum* PO27 yeast strain using a statistical design was investigated. The study was conducted through 12 runs to investigate the impact of the chosen variables. The outcomes of the selection experiments, along with the corresponding alpha-amylase activity, are presented in Table 10, using the Plackett-Burman design.

Table 10: Plackett-Burman design matrix of 12 runs and related responses.

Run	A	B	C	D	E	F	G	H	I	J	K	α-amylase activity U/g
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	**100.31**
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	**106.75**
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	**92.26**
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	**152.39**
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	**116.83**
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	**100.57**
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	**108.84**
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	**132.32**
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	**148.41**
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	**116.63**
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	**139.83**
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	**117.77**

The model's effectiveness is illustrated by the R^2 value of 0.9726, indicating that the model can account for 97.26 % of the response variability, and adjusted determination coefficient (R^2_{adj}) was 89.96%. Table 11 displays the statistical analysis of the responses. The model's F-value of 13.31 signifies its significance. Prob values of 0.028 suggest that the model terms hold significance.

Table 11: Statistical analysis for PBD.

Code	Effect	Coef	T-Value	F-Value	P-Value
Model				**13.32**	**0.028**
Constant		119.41	66.35	-	0.000
Soluble starch	0.510	0.92	0.51	0.26	0.645
Malt extract	3.737	6.73	3.74	13.97	**0.033**
NH_4Cl	0.394	0.71	0.39	0.16	0.720
pH	0.786	1.42	0.79	0.62	0.489
Moisture content	-6.243	-11.24	-6.24	38.98	**0.008**
Inoculum	-0.670	-1.21	-0.67	0.45	0.551
$CaCl_2$	6.843	12.32	6.84	46.84	**0.006**
$MgSO_4$	-2.302	-4.14	-2.30	5.30	0.105
Error					

A, B, C, E, F, G, I, and **J** denote the components of the medium, while **D, H**, and **K** represented the dummy variables. For a significance probability of at least 70%, variables with a probability equal to or exceeding 70% are accepted and recognized as having an influence on the response, specifically, the production of α-amylase. The regression analysis indicated that the α-amylase level was significantly influenced by only three independent variables (Figure 20). Among these three independent variables, the percentage of calcium chloride ($CaCl_2$) had the most substantial impact on α-amylase production, with an effect of 6.843 (91.66%) which concurs with Gangadharan *et al*'s study(2008), which indicate that Ca^{2+} ions supplementation enables good growth and better α-amylase production and it is necessary to uphold the spatial conformation of the enzyme. Consequently, Ca^{2+} ions play a crucial role in maintaining enzyme stability. In contrast to the results obtained by Effiom and Lennox (2022), where the addition of $CaCl_2$ showed no effect on enzyme activity and was deemed unnecessary, Their findings indicate that Ca^{2+} neither inhibited nor potentiated α-amylase activity. This suggests that the amylase from *Streptomyces* strain 20r is calcium-independent, making it a noteworthy candidate for starch liquefaction, particularly in the production of fructose syrup.

Following this, the moisture content of the production medium demonstrated a significantly negative effect (-6.2436) which suggests that beyond a certain level of moisture content, catabolic repression may occur ,leading to a decrease in α-amylase activity, as described by Premalatha *et al.* (2022) that the solid-state fermentation (SSF) process differs from submerged fermentation (SmF) as microbial growth occurs at or near the surface of solid substrate particles with low moisture content. Therefore, it is crucial to provide optimized water content, and equally important to control the water activity of the fermenting substrate, as both lower and higher water levels can impact microbial activity.

Malt extract exhibited an effect of 3.737, contributing to 79.76% of the influence. Previous studie employing PBD have also emphasized the significance effect of this as nitrogen source in α-amylase production, Swetha *et al.*(2014) reported its negative significance effect on α-amylase production by *Piriformospora indica.* While magnesium sulfate ($MgSO_4$) (-2.302) (20.23%), and inoculum size (-0.670) (32.14%) had a negative insignificant effect on α-amylase production. In contract with Jha *et al.* (2013) who reported maximum amylase production by an isolate belonged to the *Aspergillus* genus in presence of Mg^{2+} but little $Ca^{2}+$. Also results from Effiom and Lennox' study , (2022) show directly the effectiveness of MgSO4 in amylase production from *Bacillus subtilis* IMD34 using PBD . Kammoun *et al.* (2008) shows that among the variables with the greatest effect on α-alpha amylase production by *Aspergillus oryzae* CBS 819.72 is the linear term inoculum size.

The starch variable, considered the main factor influencing α-amylase production according to the literature (Effiom and Lennox, 2022 and Ensari *et al.*, 1995),and it was found to have no significant effect on enzyme production, with an estimated p-value of 0.645, effect of 0.510, 55.96 percent. In contrast to our findings, the optimal level of amylase from *Bacillus* sp. was achieved when starch was utilized as the sole carbon source (Khusro *et al.*, 2017). In fact, the statistical study revealed no effect of pH on alpha-amylase production, which concurs with the results of Gangadharan *et al.* (2008), who found that extracellular α-amylase synthesis is not affected by the pH of the medium. On top of that, there is no significant effect of ammonium chloride (NH_4Cl) on α-amylase production by *G. candidum* PO27 (0.39407) (44.04%) as shown in Pareto Chart of the standardized effects (Figure 21). The paper by Akcan *et al.* (2009) demonstrates that there is no significant increase in the yield of α-amylase produced by *Bacillus subtilis* RSKK96 with the supplementation of either inorganic or organic nitrogen sources.

The highest activity was observed in run 4 at 152.39 U/g, where all the significant medium components ($CaCl_2$, Malt extract) were present at their maximum levels expect the moisture content at their minimum level due to their significant negative effect on the production. In contrast, run 3 exhibited the lowest activities 92.26 U/g, this discrepancy could be attributed to the absence of significant variables, despite the presence of inducers (starch) in run 10.

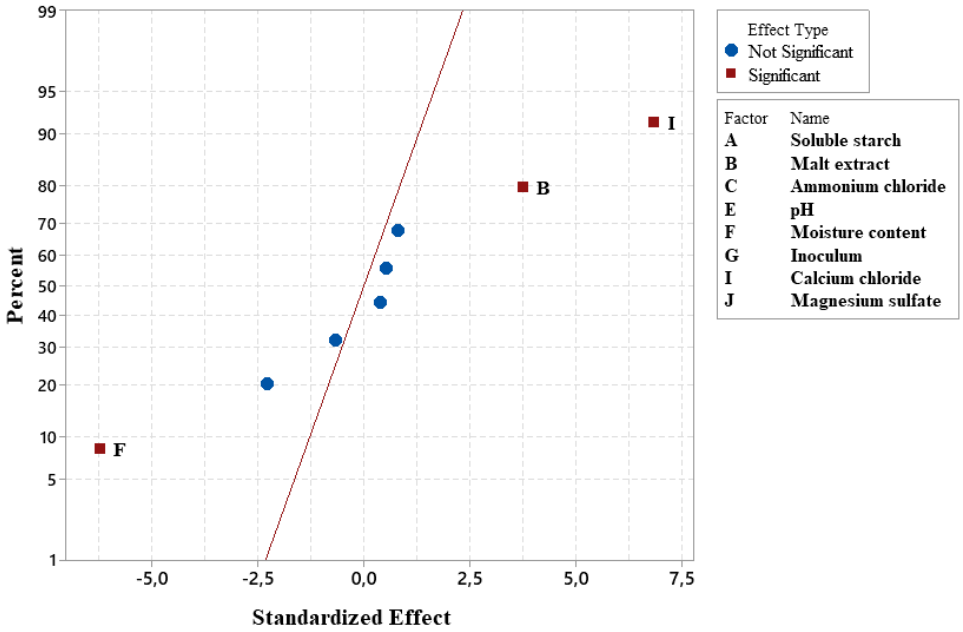

Figure 20: Normal plot of the standardized effects (response is Response; α = 0,05).

NOVA revealed the p-values of calcium chloride, moisture, and malt extract content to be <0.006, 0.008, and 0.033, respectively (<0.05), indicating these as the most significant variables influencing α-amylase production by *Geotrichum candidum* PO27. The results were subjected to regression analysis, resulting in the development of a first-order polynomial equation that describes alpha-amylase production based on the independent variables:

$$\mathbf{Y} = 119.41 + 0.92\,A + 6.73\,B + 0.71\,C + 1.42\,E - 11.24\,F - 1.21\,G + 12.32\,I - 4.14\,J$$

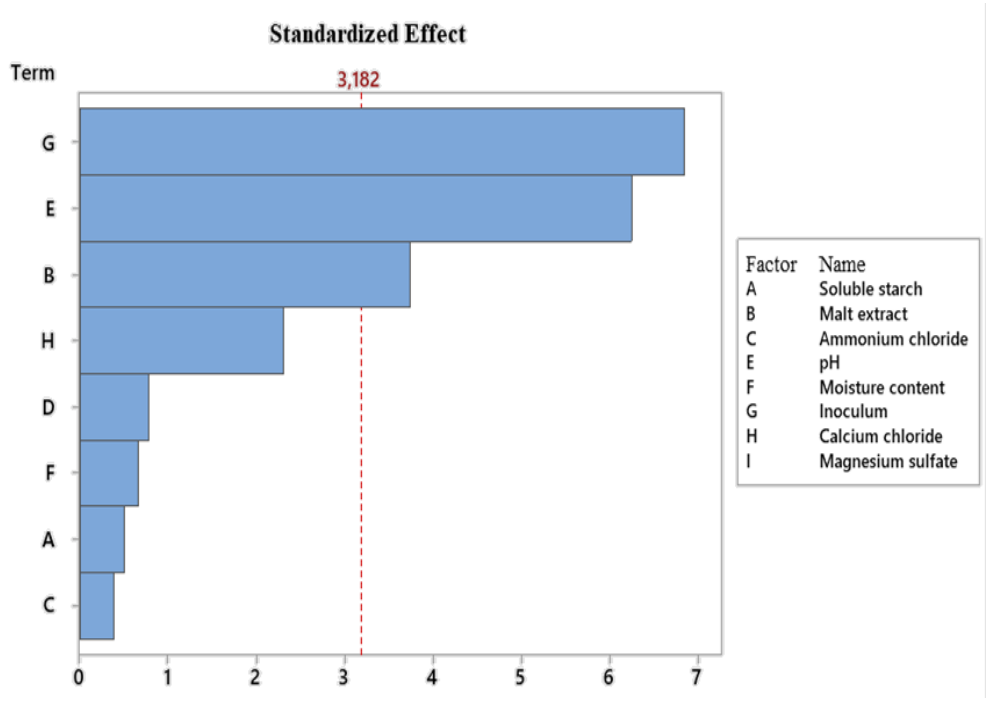

Figure 21: Pareto Chart of the Standardized Effects (response is Response; α = 0,05).

6.2. Central composite design (CCD) for optimization of α-amylase production

Building on the outcomes of the initial screening processes, which highlighted the significance of three test variables (malt extract, moisture content, and calcium chloride) in α-amylase production by *G. candidum* PO27, we advanced to optimize these parameters using Response Surface Methodology (RSM) with a central composite design (CCD). A CCD consisting of 20 experiments was conducted, comprising 8 factorial experiments, 6 axial experiments, and 6 central experiments, as previously mentioned. The details of the 20-run CCD, along with the corresponding α-amylase activity ranging from 103.390 to 225.630 U/g, are provided in Table 12.

Table 12: Central composite design matrix of 20 runs and related responses**.**

Run	Variable Levels			α-amylase activity (U/g)
	A	**B**	**C**	177.750
1	-1	-1	-1	192.140
2	+1	-1	-1	178.100
3	-1	+1	-1	121.110
4	+1	+1	-1	168.715
5	-1	-1	+1	**225.630**
6	+1	-1	+1	121.330
7	-1	+1	+1	146.735
8	+1	+1	+1	141.120
9	-1.682	0	0	123.805
10	+1.682	0	0	**221.260**
11	0	-1.682	0	146.060
12	0	+1.682	0	176.640
13	0	0	-1.682	**210.470**
14	0	0	+1.682	103.390
15	0	0	0	121.380
16	0	0	0	109.500
17	0	0	0	110.565
18	0	0	0	114.075
19	0	0	0	112.060
20	0	0	0	177.750

The CCD shown in Table 13 had an F-value of 25.15 and a p-value of < 0.05, implying the significance of the studied model. The F-value and p-value serve as indicators of the effect of the terms involved on the response. A larger F-value and a smaller p-value signify higher significance (Mehmood *et al.*, 2018). This suggests that the experimental data obtained are in good agreement with the model. The multiple regression analysis of the experimental data led to the formulation of a second-order quadratic polynomial equation for α-amylase activity, expressed as follows:

Y= 112.05 + 1.30 A - 39.83 B + 6.18 C + 16.48A^2 + 67.68 B^2 + 77.57 C^2 – 36.4 AB + 42.2 AC – 19.7 BC.

Y is the α-amylase activity, A, B and C are the values of the test variables; A: malt extract (g %), B: Moisture content (v/w), C: $CaCl_2$ (g %).

Statistical analysis indicates coefficients of determination of R^2 = 0.9577, meaning that 95.77 % of the variation in α-amylase production can be explained by the three variables studied. The R^2 value of more than 0.9, signifies a robust correlation and close alignment between the experimental and predicted values (Krishnankutty, 2024), which means that the model chosen is suitable for production.

Table 13: Regression coefficient for the α-amylase production equation.

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	112.05	4.61	**24.31**	**0.000**	
Malt extract	1.30	5.14	0.25	0.805	1.00
Moisture content	-39.83	5.14	-7.74	0.000	1.00
Calcium chloride	6.18	5.14	1.20	0.257	1.00
Malt extract*Malt extract	16.48	8.42	1.96	0.079	1.02
Moisture content*Moisture content	67.68	8.42	8.04	0.000	1.02
Calcium chloride*Calcium chloride	77.57	8.42	9.21	0.000	1.02
Malt extract*Moisture content	-36.4	11.3	-3.22	0.009	1.00
Malt extract*Calcium chloride	44.2	11.3	3.91	0.003	1.00
Moisture content*Calcium chloride	-19.7	11.3	-1.74	0.113	1.00

Results of ANOVA showed the effect of different parameters and their interactions represented in the Fischer test at 5% significance level (Table 14). The linear term- moisture content (B); interactive terms malt extract and moisture content (AB), malt extract and $CaCl_2$ (AC) and quadratic terms- moisture content (B^2), $CaCl_2$ (C^2); showed p-values < 0.05 and were the significant ones affecting α-amylase production. The interaction between moisture content and $CaCl_2$ (BC) with a p-value of 0.113 was insignificant.

Table 14: Analysis of Variance (ANOVA) generated by CCD model for optimization of α-amylase production.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	28913.7	3212.6	**25.15**	**0.000**
Linear	3	7852.5	2617.5	20.49	0.000
Malt extract	1	8.2	8.2	0.06	0.805
Moisture content	1	7659.7	7659.7	**59.97**	**0.000**
Calcium chloride	1	184.6	184.6	1.45	0.257
Square	3	17400.9	5800.3	**45.41**	**0.000**
Malt extract*Malt extract	1	489.2	489.2	3.83	0.079
Moisture content*Moisture content	1	8249.9	8249.9	**64.59**	**0.000**
Calcium chloride*Calcium chloride	1	10838.7	10838.7	**84.86**	**0.000**
2-Way Interaction	3	3660.3	1220.1	9.55	0.003
Malt extract*Moisture content	1	1323.3	1323.3	**10.36**	**0.009**
Malt extract*Calcium chloride	1	1950.6	1950.6	**15.27**	**0.003**
Moisture content*Calcium chloride	1	386.4	386.4	3.03	0.113
Error	10	1277.2	127.7		
Lack-of-Fit	5	1102.6	220.5	6,32	0.032
Pure Error	5	174.6	34.9		
Total	19	30190.9			

Considering the results presented in Pareto chart (Figure 22), shows the influence of variances and their interactions on alpha amylase production. The factor C^2 (Calcium chloride*Calcium chloride) exhibits the most significant effect with a value of 9.21, contributing to 92.53%. Following closely is B^2 (Moisture content*Moisture content) with an

effect of 8.037 (81.91%), B (Moisture content) with 7.744 (74.44%), AC (Malt extract*Calcium chloride) with 3.908, and AB (Malt extract*Moisture content) with 3.218 (18.08%).

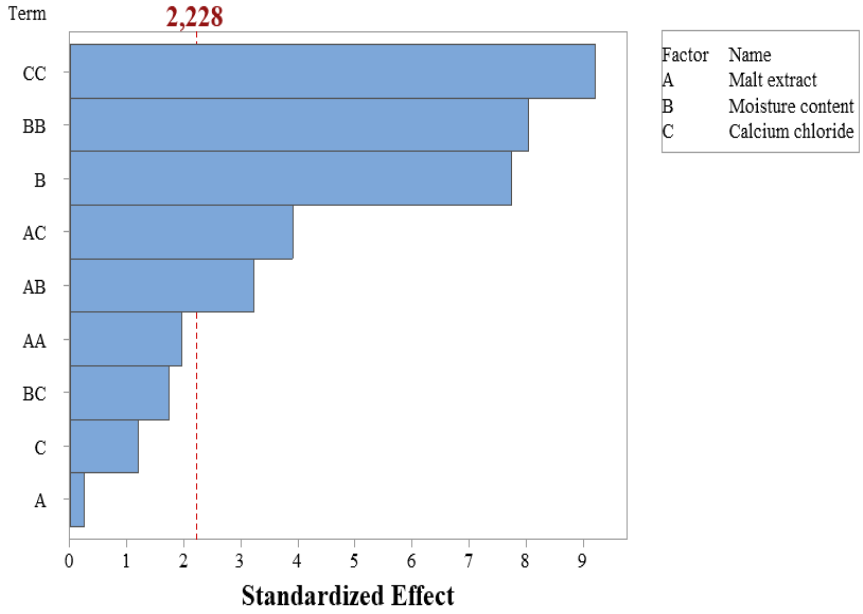

Figure 22: Pareto Chart of the standardized effects (response is α- amylase activity (U/g); α = 0.05).

The quantitative effect and interaction between the most effective factors $CaCl_2$ malt extract, and moisture content α-amylase production determined from PB design were shown by Contour plots (Figure 23). Each contour plot represents an infinite number of combinations of two test variables with the other held at their respective zero level. It's provide a visual interpretation of the interaction between the two variables, and make it easier to locate the optimum experimental conditions. According to the graph, enzyme production was effectively increased at the extreme values of the factors. The red areas indicate the conditions that optimize enzyme production.

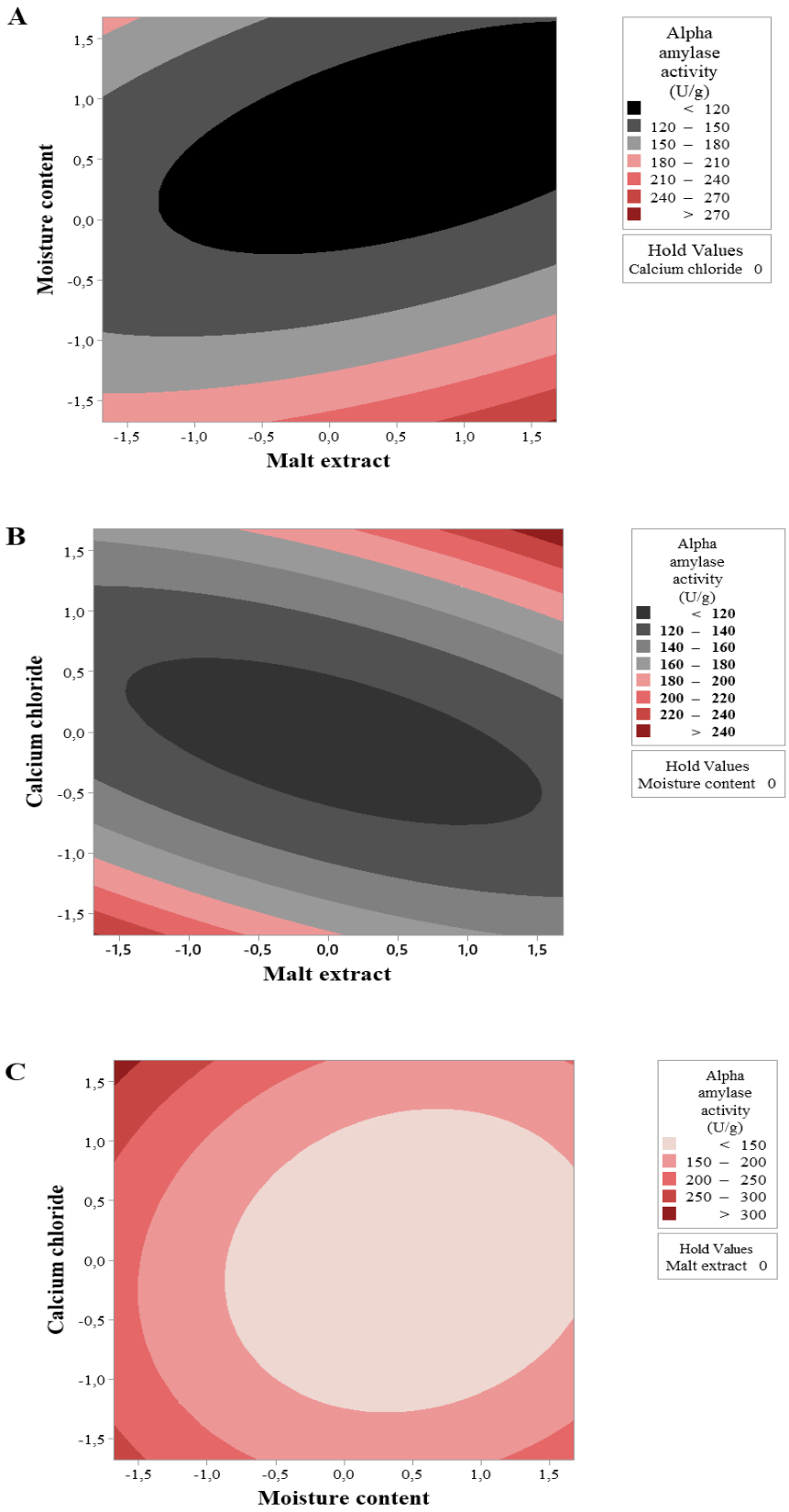

Figure 23: 2D Contour Plot of α- amylase from *G.candidum* PO27 showing interactions between A: Moisture content; Malt extract, B: Calcium chloride; Malt extract, C: Calcium chloride; Moisture content.

The 3D response surfaces serve as graphical representations of the regression equation for α-amylase yields. Figures 6, 7, and 8 demonstrate the effects of interactions between malt extract and moisture content, $CaCl_2$ and moisture content, and malt extract and $CaCl^2$ on α-amylase production. These plots provide a straightforward and convenient means to comprehend the interactions between parameters and identify their optimal levels **(**Figure 24).

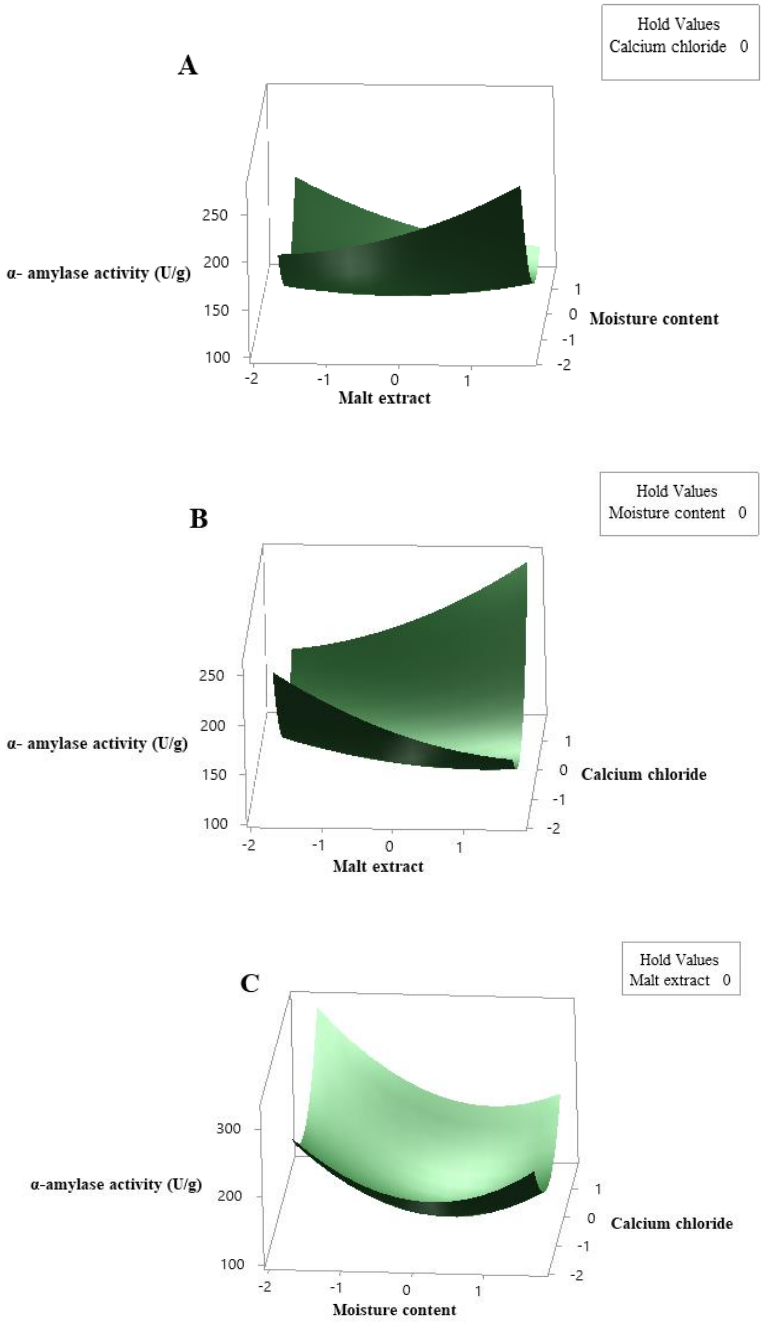

Figure 24: Surface plots of α-amylase production by *Geotrichum candidum PO27* strain (A: Moisture content; Malt extract, B: Calcium chloride; Malt extract, C: Calcium chloride; Moisture content).

6.2.1. Response optimization

To determine the optimized parameters, a systematic investigation of the response surface analysis between the input and output responses is necessary. The optimization results, as illustrated in Figure 25, indicate that the optimal parameters are approximately: moisture content 24.77%, malt extract 1.84 g % and $CaCl_2$ 1.84 g %. The corresponding mechanical performance can be predicted accordingly. To validate the accuracy of the prediction, a verification experiment should be conducted under the optimal parameters.

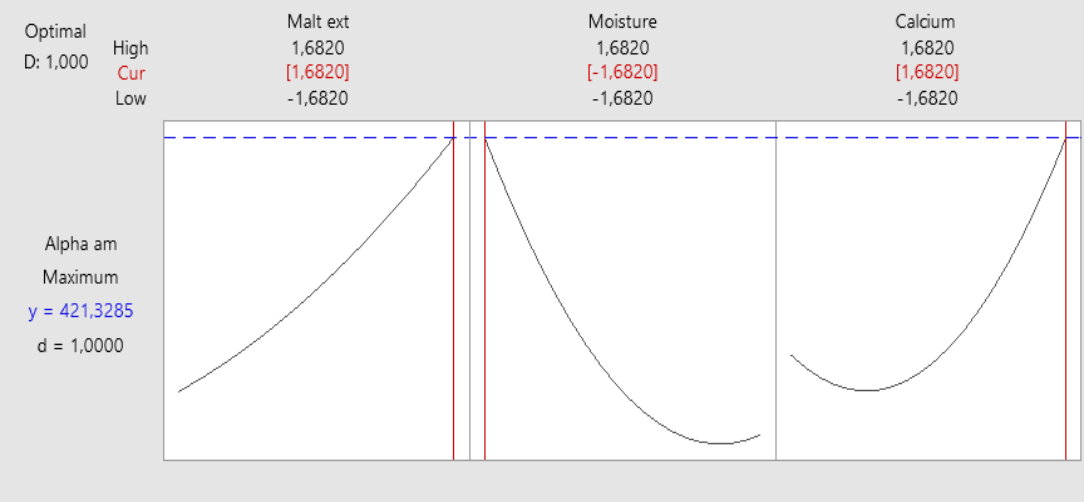

Figure 25: Response optimizer plot.

6.2.2. Validation of the model

The aim of statistical optimization was to maximize enzyme activity within the defined variable range. As per Minitab v19, predict the highest desirability and response for the enzyme production. The predicted levels of significant factors resulting in a response of 421.32 U/g (Figure 23) were moisture content 24.77%, malt extract 1.84 g % and $CaCl_2$ 1.84 g %. The experiment was conducted in duplicate, and the mean experimental response of 412.94 U/g aligned closely with the predicted response, indicating a strong correlation and validating the model.

7. Kinetics for α-amylase production

The production of alpha-amylase by the *G. candidum* PO27 strain occurs in 250 ml Erlenmeyer flasks utilizing the optimized olive pomace medium through solid-state fermentation. In Figure 24, the kinetics and monitoring of enzyme production are illustrated over a 100-hour period. The alpha-amylase production varies with incubation time as follows:

- [0-24h]: The activity remains stable from the start of fermentation.
- [24-40h]: A progressive increase is observed, reaching a peak level of 373.15 U/g at 40h, representing two-and-a-half-fold increase (2.5 fold) compared to the start of fermentation activity (at 4h).
- [40-48h]: A drop to 133.35 U/g is noted during this period.
- [48-100h]: The average amylolytic activity experiences a slight increase at 56h (156.44 U/g) and 72h (169.93 U/g), followed by a gradual decrease to a minimum level of 47.65 U/g after 100h of fermentation.

The total protein amount varies throughout the fermentation process, reflecting both protein consumption and production (Figure 26). The maximum value, reaching is achieved after 68 hours of fermentation. This increase can be attributed to protein synthesis, including the synthesis of enzymes and other proteins (Coman *et al.*, 2012 and Kamilari *et al.*, 2023).

The sugar consumption rate is notably high during the initial 16 hours, attributed to the utilization of polysaccharides present in the olive pomace by the strain (Cardoso *et al.*, 2002 and Miranda *et al.*, 2019). This is consistent with its ability to degrade the lignocellulosic substrate (olive pomace) (Leite *et al.*, 2016) through the production of various enzymes, including cellulase. Its products are utilized in the growth phase and may act as inducers for α-amylase production, as evidenced by the observed peak following this period (40h). Subsequently, from hours 16 to 40, a gradual increase in sugar concentration is observed, likely a result of polysaccharide production by *G. candidum* PO27 (Van Bogaert *et al.*, 2009) .

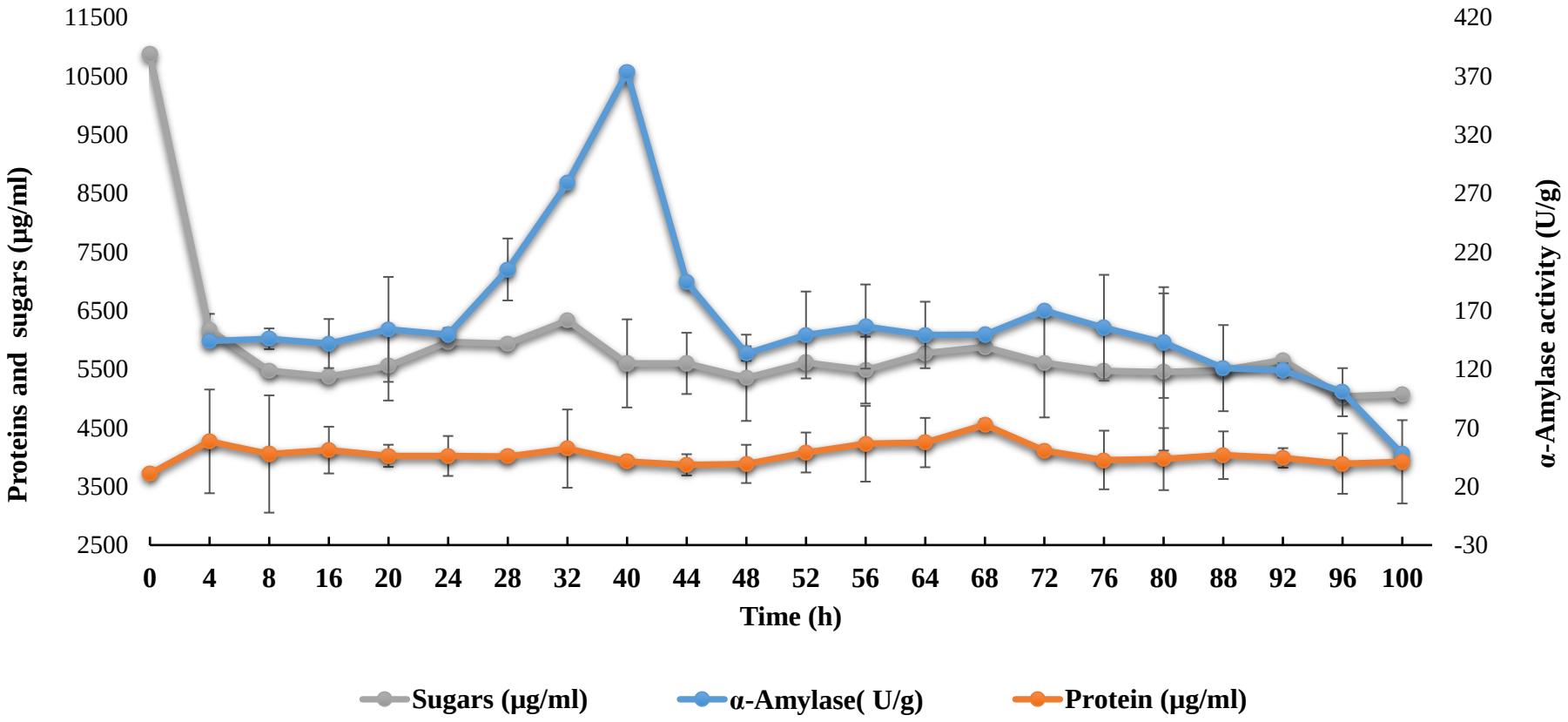

Figure 26: Kinetic evolution of α-amylase production, sugars and proteins by the isolated yeast *G. candidum* PO27 grown on optimized olive pomace waste medium in 250 ml Erlen Meyer flasks

The pH evolution profile is depicted in figure 27, revealing four distinct phases. The initial phase corresponds to the onset of growth, lasting approximately 4 hours, with a slight increase in pH from 6.18 to 6.59. In the second phase, pH undergoes a rapid decrease (within 10 hours) from 6.59 to 5.33 due to the active growth of the mycelium. This growth releases organic acids, progressively acidifying the medium (Punia Bangar *et al.*, 2022). In the third phase, the pH remains consistently within the range of 5.53 to 5.58 for over 56 hours. Afterward, there is an increase to 5.74 by the 64th hour of fermentation. Finally, in the last phase, the pH decreases and remains stable until the end of the culture.

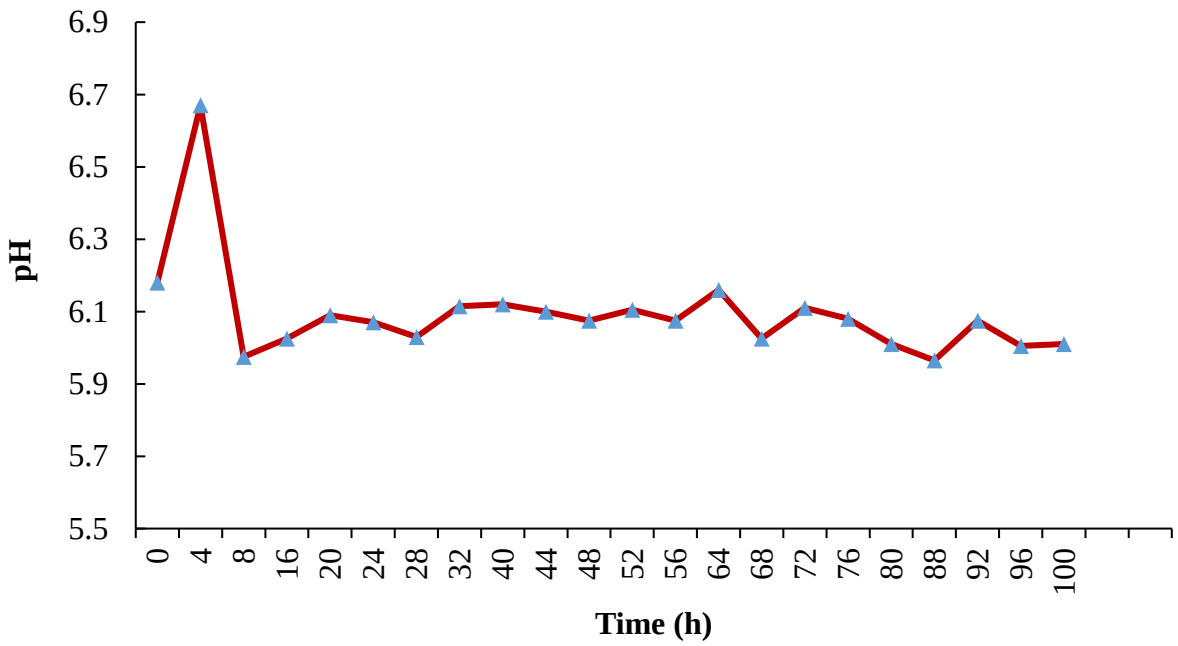

Figure 27: The pH evolution profile.

8. Enzyme purification

After elution with pH 7 Tris Hcl buffer (20mM), five peaks appeared and the corresponding fractions were grouped together. The α-amylase activity assay showed that the best activity was found in peak 5 (Figure 28) with fractions from 34 to 38 with a total volume of 7.5 ml and 21.69 U/ml of amylase and a total activity of 162.72 U/ml. The specific activity increased from 856.27 U/mg to 5764.54 U/mg. The yield was 9.11% with a purification factor of 6.73. The purification steps are shown in Table 15.

Table 15: Purification steps.

Purification steps	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crud extract	1784.754	2.084	856.271	1	100
Ultrafiltration 10k	1633.513	0.453	3599.863	4.204	91.525
Ultrafiltration 5k	491.292	0.122	4015.652	4.689	27.527
Superdex 75 column	162.728	0.028	5764.540	6.732	**9.117**

A previous study on α-amylase purification from *Geotrichum candidum* isolated from soil reported the separation of two alpha amylase enzymes with a yield of 10.9% for the enzyme that may degrade soluble potato starch and a 2% yield for the degradable alpha amylase from raw starch (Attanayaka *et al.*, 2009).

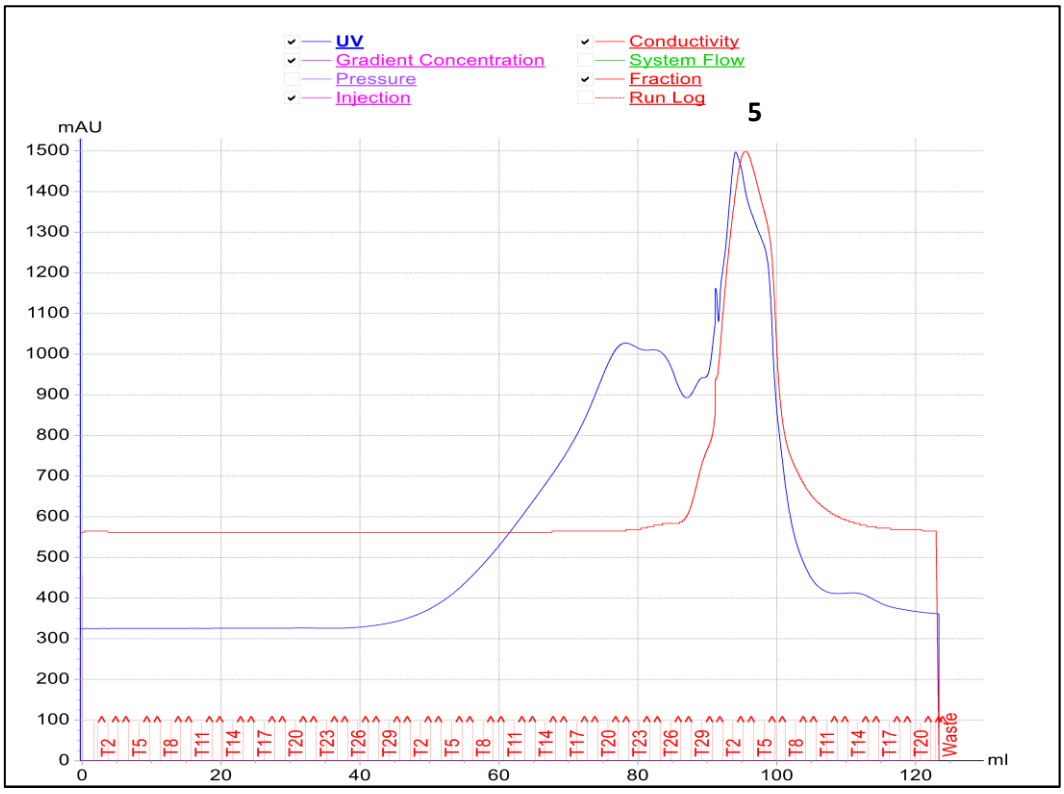

Figure 28: Elution profile of α-amylase using exclusion chromatography.

9. SDS page electrophoresis

The protein bands were subjected to staining by Coomassie brilliant blue R-250 to make them visible. SDS-PAGE showed that the molecular mass of the α-amylase from *Geotrichum candidum* PO27 was around 19.2 kDa (Figure 29). The molecular weight in the present study is close to that of α-amylase (20 kDa) from *Bacillus caldolyticus* that exhibited the lowest molecular weight reported by Gupta e*t al.* (2003). Similar to our results, Roy *et al.* (2012), investigated the production of amylase from *B. subtilis* AS-S01a having a molecular weight of 21 kDa.

Microbial amylases showed a wide range of molecular weight from 10 to 210 kDa (El-Enshasy *et al*., 2013). The molecular weight of α-amylase from *G. candidum* PO27 is substantially higher than alkaline thermostable α-amylase weight produced from *Geobacillus* sp. nov. which was 12.2 kDa (Febriani *et al.*, 2019), and higher from *Pseudomonas stutzeri* amylase (12.5kDa) isolated by Robyt and Ackerman (1971) , and lower than other microbial α-amylases, which generally have molecular weight well above 20 kDa. The one produced by bacterial strain sps2 even has a molecular weight of 28 kDa (Saha *et al.*, 2023).

A variety of bacilli have different α-amylase molecular masses of 43, 50, 60.5-86, 91 and 97 kDa, respectively, for *Anoxybacillus beppuensis* TSSC-1, *Anoxybacillus* sp. SK3-4, *Bacillus* sp. A3-15, *Bacillus* sp. AAH-31, and *Geobacillus* sp. IIPTN (Acer *et al*., 2016).

α-Amylase's molecular weight was found as 74 kDa from thermophilic *Bacillus licheniformis* SO-B3 (Fincan *et al.*, 2021) , as well as that of another α-amylase from thermophilic *Anoxybacillus thermarum* A4 strain is 50 kDa, by Baltas *et al.* (2016).

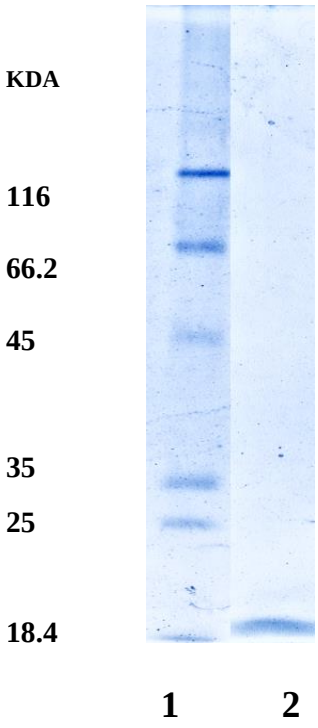

Figure 29: SDS-PAGE electrophoretic assay of purified enzyme (1: Protein ladder, 2: purified *G. candidum* PO27 α-amylase).

10. Glycosylation rate of the pure α-amylase

Glycosylation is a process by which carbohydrates are added to proteins or lipids to form glycoproteins or glycolipids. This process enhances the enzymes' stability and may have a considerable effect on their physico-chemical and biological properties. (Srimathi and Jayaraman, 2005). The carbohydrate content of the purified *G. candidum* PO27 *α-amylase* was evaluated to be 246.91µg/ ml; this result indicated its glycoprotein nature.

11. Characterization of the purified α-amylase

11.1. Effect of temperature

The impact of temperature on the purified α-amylase activity was assessed within the range of 30°C to 100°C. The optimal α-amylase activity was determined at 70°C (p=0.000), at temperatures more than 80°C, a little loss in the amylase activity was observed (Figure 30.A). Similar findings have been reported for the α-amylase from *Bacillus* sp. isolate where the enzyme completely lost its activity at temperatures greater than 50°C (Singh *et al.*, 2016). Optimal temperature for amylase produced by *Bacillus velezensis* was approximately 70 °C (Zhang *et al*., 2021). However, *Aspergillus niger* and *Aspergillus oryzae* amylases showed maximum activity at 40 °C and 55 °C respectively (Balakrishnan *et al*., 2021 and Mahmood *et al*., 2018).

The enzyme thermostability was studied after incubation for 30, 60, 90, 120, 150 and 180 min at two different temperatures (70 and 100°C), and pH 5 buffer.

According the Figure 30.B, it appears that the enzyme activities maintained at 70 and 100°C was found 19.53% and 18.70 % respectively, after incubation of 180 min. As for the alpha amylase half-life, it is 54% at 70°C and 58% at 100°C after 1h. Similar to Kalia *et al.* (2021) , when partially purified α-amylase from *Trichoderma reesei* was stable for 1 h at 70°C and retain 45% residual activity , contrary to Finore *et al.* (2011) , the residual activity was only 10% when the α-amylase from *Geobacillus thermoleovorans* subsp. *stromboliensis* subsp. nov was incubated at 70°C for 24 h. Also to Niyomukiza *et al.* (2023) who found that after pre-incubation at 70°C for 60 minutes, the alkaline α-amylase from *Bacillus subtilis* strain W3SFR5 's original activity was maintained at 80%.

Various studies of α-amylases from thermophilic microorganisms have shown that they have a lower optimum temperature with greater thermal stability than that obtained in our study. Thermophilic *Bacillus* sp. α-amylase was stable for 2 h at temperatures ranging from 40 to 50°C (Cordeiro *et al.*, 2002). Also the α- amylase from *Aspergillus tamarii* MTCC5152 is relatively stable in the temperature range of 25–35 °C for a period of 4 h hence (Arunachallam *et al*., 2023). However, the amylase produced from the isolated bacterium M13 retained 80.94% of its initial activity after incubation at 80°C for an hour.

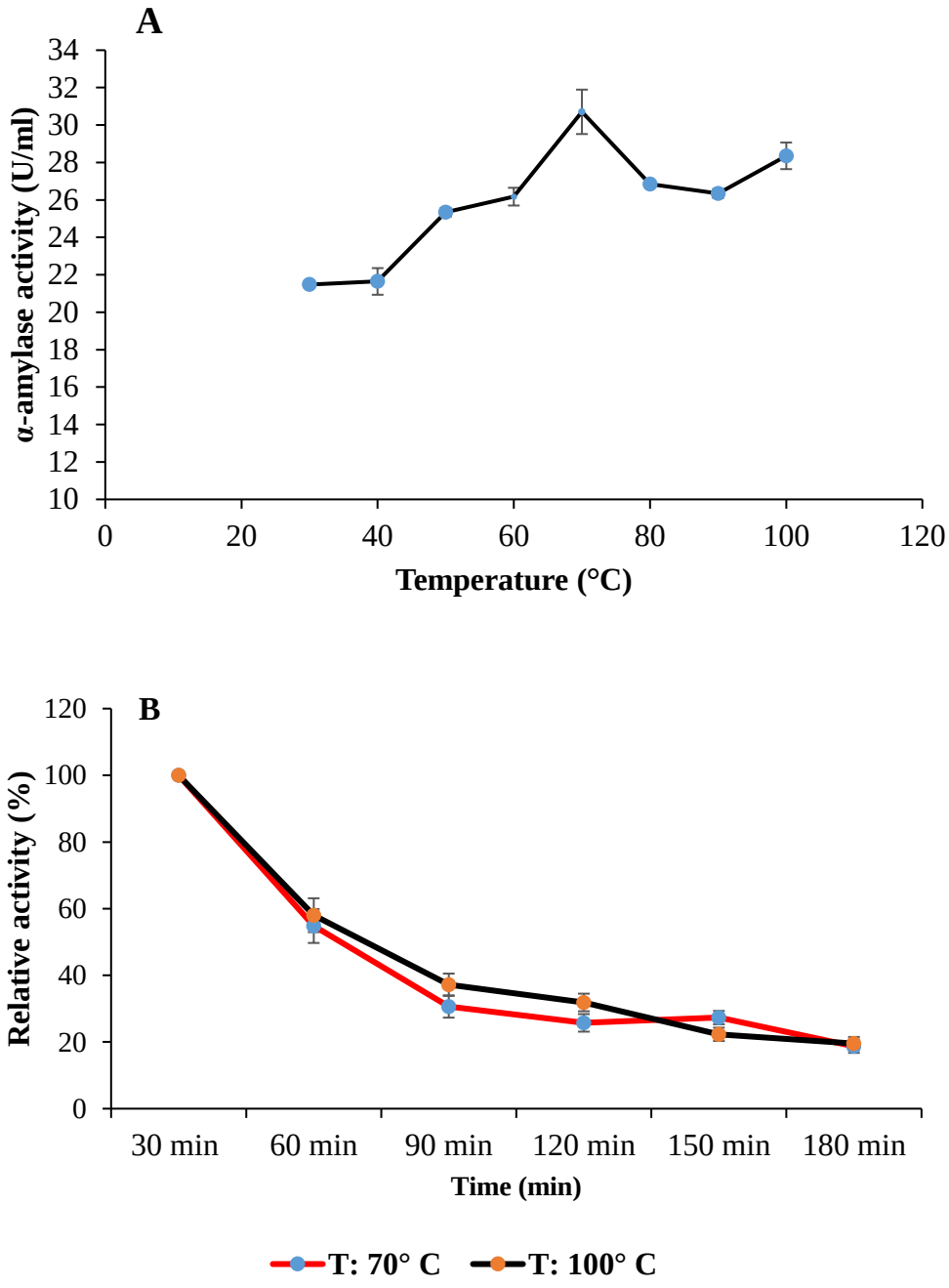

Figure 30: Effect of temperature on α-amylase activity (A: Optimum temperature ; B: Temperature stability of purified α-amylase for 70 and 100°C).

11.2. Effect of pH

During the study of the physicochemical parameters of α-amylase, the pH effect on the enzyme activity is studied and showed in Figure 31. The activity of purified *G. candidum* PO27 α- amylase is tested over a pH range from three to 11. The optimum pH was found at 5 (22,48U/ml) (p=0.000), while in the pH range from 6 to 11, the activity was lower. Similar results were presented by some researchers (Fincan *et al.*, 2021) who found that the pH optimum α-amylase isolated from *Bacillus licheniformis* So-B3 is from pH 5 to 6. Our obtained data showed also a good agreement with the studies of Sethi *et al.* (2016) ,when *Aspergillus terreus* NCFT 4269.10 α-amylase had an optimum of pH 5.

Also, similar results were obtained with α-amylase produced from fungi such as *Aspergillus niger* (Bagheri *et al.*, 2014) and from *Trichoderma reesei* (Kalia *et al.*, 2021) with maximum activity at pH 5 indicating acidophilic nature of enzyme, whereas Mahmood *et al.* (2018) reported maximum α-amylase activity at pH 6 and Attanayaka *et al.* (2009) showed maximum activity at pH 7 of *Geotrichum candidum* CMSS06 purified α-amylase.

It was indicated that an optimal pH of thermostable α-amylase is very common in the range of 5 to 6 (Oyedeji *et al.*, 2023). In contrast with Saha *et al.* (2023), the purified α-amylase from actinobacteria *Streptomyces pratensis* sps2 remained active in high pH value than in lower pH .

From the results, we can therefore conclude that purified α-amylase is acidic in nature and can be applied in various industrial processes such starch industries, where acidic conditions are required.

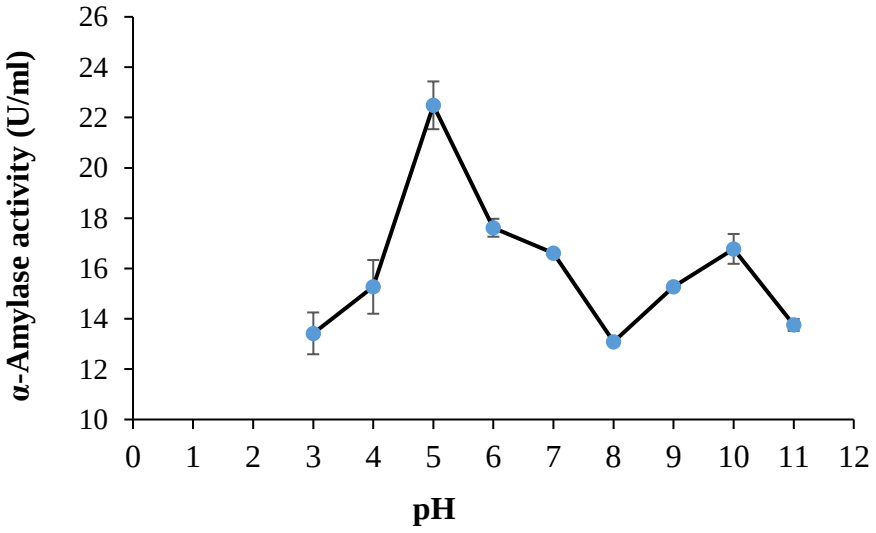

Figure 31: Effect of pH on α-amylase activity.

11.3. Determination of the kinetic parameters

Figure 32 illustrated the results of kinetic investigations, conducted on the purified enzyme, employing diverse concentrations of soluble starch as the substrate within optimum assay conditions. Through the Line weaver–Burk plot, the determined **K_m** and **V_{max}** values were 0.114 mg/ml and 588.23 U/ml, respectively at 70°C.

Comparing the kinetic parameters K_m and V_{max} of various amylases proves challenging due to differences in substrate origin, enzyme source, and enzyme assay methodology. In the realm of enzyme kinetics, the K_m and V_{max} serve as crucial coefficients. A smaller K_m value indicates a higher affinity of the enzyme for the substrate, as elucidated by Hamilton *et al.* in 1999. In a related investigation, Saha *et al.* (2023) presented V_{max} and K_m values for alpha-amylase from *Streptomyces pratensis sps*2 , recorded at 2 mg /ml and 1000 U/min, respectively, at 40°C. Egbune *et al.* (2022) documented K_m and V_{max} values for alpha-amylase from *Rhizopus oligosporus* at 16.39 mg/ ml and 37.59 U/mg, respectively. Additionally, Kizhakedathil and C, (2021) reported K_m and V_{max} values for α-amylase from *Pseudomonas balearica VITPS19 as* 45.23 mg ml^{-1} and 20.83U/ml respectively.

The α-amylase obtained in this present study exhibits a low K_m and a higher V_{max} value, signifying its catalytic efficiency. This characteristic suggests that the enzyme could be more effectively utilized in starch degradation processes (Saha et *al.*, 2023).

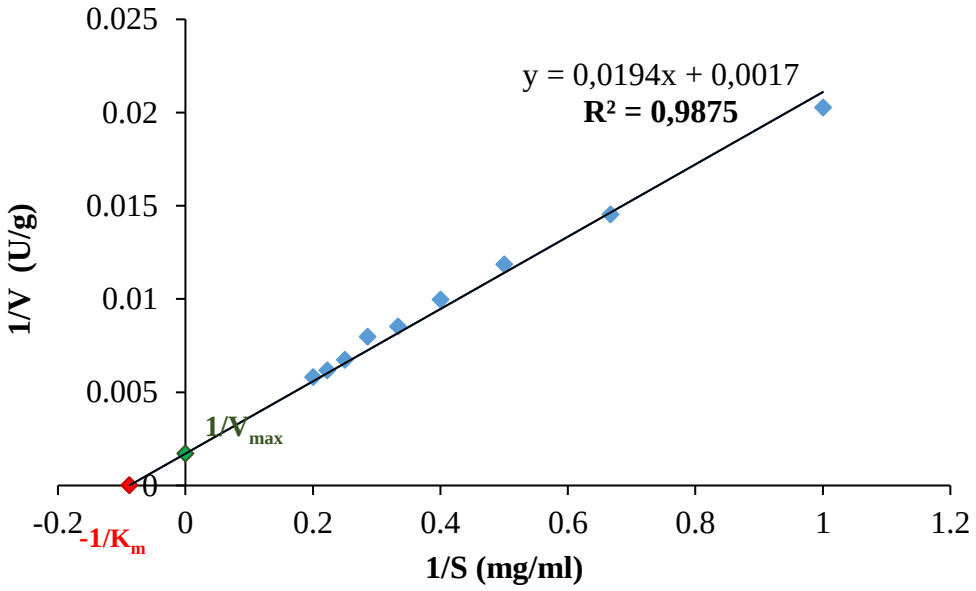

Figure 32: Lineveaver and Burk representation of purified *G. candidum* PO27 α-amylase.

12. Effect of different metals and chemical reagents

12.1. Metal ions

It has been largely demonstrated that metal ions are important in the regulation of enzyme activities; metal ions could play an influential factor in the stability and activity of enzymes (Unnikrishnan *et al.*, 2021), so different ions were tested for their effect on purified α-amylase from *Geotrichum candidum* PO27 (Figure 33). The ANOVA test is highly significant (P=0.000) (Annex 5). The results indicated that only 2 ions (Cu^{2+} and Mn^{2+}) seemed to have a moderate to almost total inhibitory effect (49%, 4%), agrees with the behavior observed for most α-amylases in which these heavy metals exhibited inhibitory effect, a novel halo-acid-alkali-tolerant amylase secreted from halophile *bacillus siamensis* F2 inhibited by almost 40% in the presence of $CuSO_4$ (Rathod *et al.*, 2023).Also Mn^{2+} ions completely inhibited the α-amylase enzyme produced by *Rhizobium* sp. strain , although Cu^{2+} ions reduced enzyme activity by 30% (Abdel-Hameed *et al.*, 2022). And disagrees with Egbune *et al.* (2022) who reported the effectiveness of these metals ions in the activation of α-amylase produced by *Rhizopus oligosporus* .

The inhibitory effect caused by Mn^{2+}, Cu^{2+} ions on α-amylase activity may be due to competition between exogenous cations and cations associated with the protein, leading to a decrease in activity. These ions have also been reported to inhibit other α-amylases produced by yeasts (Dakhmouche Djekrif *et al.*, 2021). A low decrease in activity was observed in the presence of Na^{+} (97%), Ni^{2+} (96%), and Zn^{2+} (93%) as compared to the control. In previous reports, most of the amylase activity was inhibited in the presence of Ni^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}. The novel α-amylase from marine *Streptomyces* sp. D1 were strongly inhibited by $Hg^{2,}$ Fe^{2+}, K^{+}.

While the *G. candidum* PO27 α-amylase activity was strongly increased with the presence of Mg^{2+} (178%) which similar results confirmed its act as an activator of α-amylase, purified α-amylase from *Bacillus pacificus* was activated in the presence of Mg^{2+} that improved enzyme activity up to 170% (Alonazi *et al.*, 2021). *G. candidum* PO27 α-amylase was slightly effected by Hg^{+} and Fe^{2+} (117%), whereas Ca^{2+} had a non-noticeable stimulating effect (104 %) which makes it even more promising for industrial application. Many amylases had Ca^{2+} cation in the active site, therefore, they are metal ion-dependent (Alonazi *et al.*, 2021) . Similar to Acer *et al*'s study (2016) who also reported that these ions have an activator effect on thermostable α-amylases produced by *Anoxybacillus* sp. AH1 whereas, Cu^{2+} exhibited an

inhibitory effect on amylase activity. In addition, the isolates MBT001 and MBT002 gave maximum activity with magnesium sulphate (Sharif *et al.*, 2023).

12.2. Chemical reagents

The effect of denaturing, chelating, inhibiting agents and effector molecules on the activity of purified PO27 α-amylase produced is as presented Figure 33.B. From the results, the ANOVA test is highly significant (p=0.000) (Annex 3), it was observed that the chelating agent (EDTA) had practically no effect on activity (97%), suggesting that the α-amylase secreted by *G. candidum* PO27 is stable to chelating agent.

Surfactants such as β-Mercaptoethanol, Tween 80, Tween 20 and Triton X-100 were studied on purified PO27 α-amylase. Both Tween 80 and Triton X-100 enhanced residual activity to 144 % and 119% respectively. Similar to Menon *et al.* (2014) who reported greater stability of amylase in surfactants from *Bacillus subtilis* strain JS-16 (Tween 80 and Triton X-100) making it a potential candidate for detergent market. Whereas β-Mercaptoethanol and Tween 20 reduced it to 55% and 85% respectively. In addition, Burhanoğlu *et al.* (2020) showed that amylase retained 66 % of relative activity in presence of Tween 20. However, partial inhibition of amylase activity was due to the disruption of protein structure by β-mercaptoethanol which break the disulfide bonds and this causes to conformational changes in the enzyme, that prevent enzyme-substrate binding and lead to loss of amylolytic activity (Nair and Bhat, 2020).

The result revealed that the purified enzyme is stable in presence of denaturing agents such as SDS and Urea at 1%. It retained 99% of its activity in presence of SDS, suggesting that the enzyme has potential in starch liquefaction and the detergent industry. On the other hand, urea slightly increases activity up to 113%. Contrary to Chakraborty *et al.* (2009) and Burhanoğlu *et al.* (2020) who found that they strongly decreased the amylase activity of the marine haloalkaliphilic strain of *Streptomyces* sp. D1 to 13.45 % ,and the novel thermostable α-amylase from *Geobacillus* sp. GS33 to 36%.

As shown in Figure 33.B, effects of several polar solvents as DMSO, ethanol and acetone were checked on purified α-amylase activity, they were found to enhance α-amylase activity for 118%, 118% and 115%, respectively. This may be interpreted as proving that the organic solvents used had no deleterious effect on the enzyme's active conformation. Whereas, Burhanoğlu *et al*'s results (2020) showed that two organic solvents such as acetone and ethanol, for example, caused inhibition and changes in protein conformation.

The results of the present study suggest that α-amylase produced during solid-state fermentation of olive pomace using *Geotrichum candidum* PO27 may be useful as an ingredient in industries that demand enzymatic starch hydrolysis, in the presence of various organic solvents and detergents. Its surfactant-stable characteristics qualify it to be helpful in the development of detergent formulations to break down the leftovers of starchy foods such as potatoes, gravies, custard, and chocolate into dextrin's and other smaller oligosaccharides (Egbune *et al.*, 2022 and Rathod *et al*., 2023).

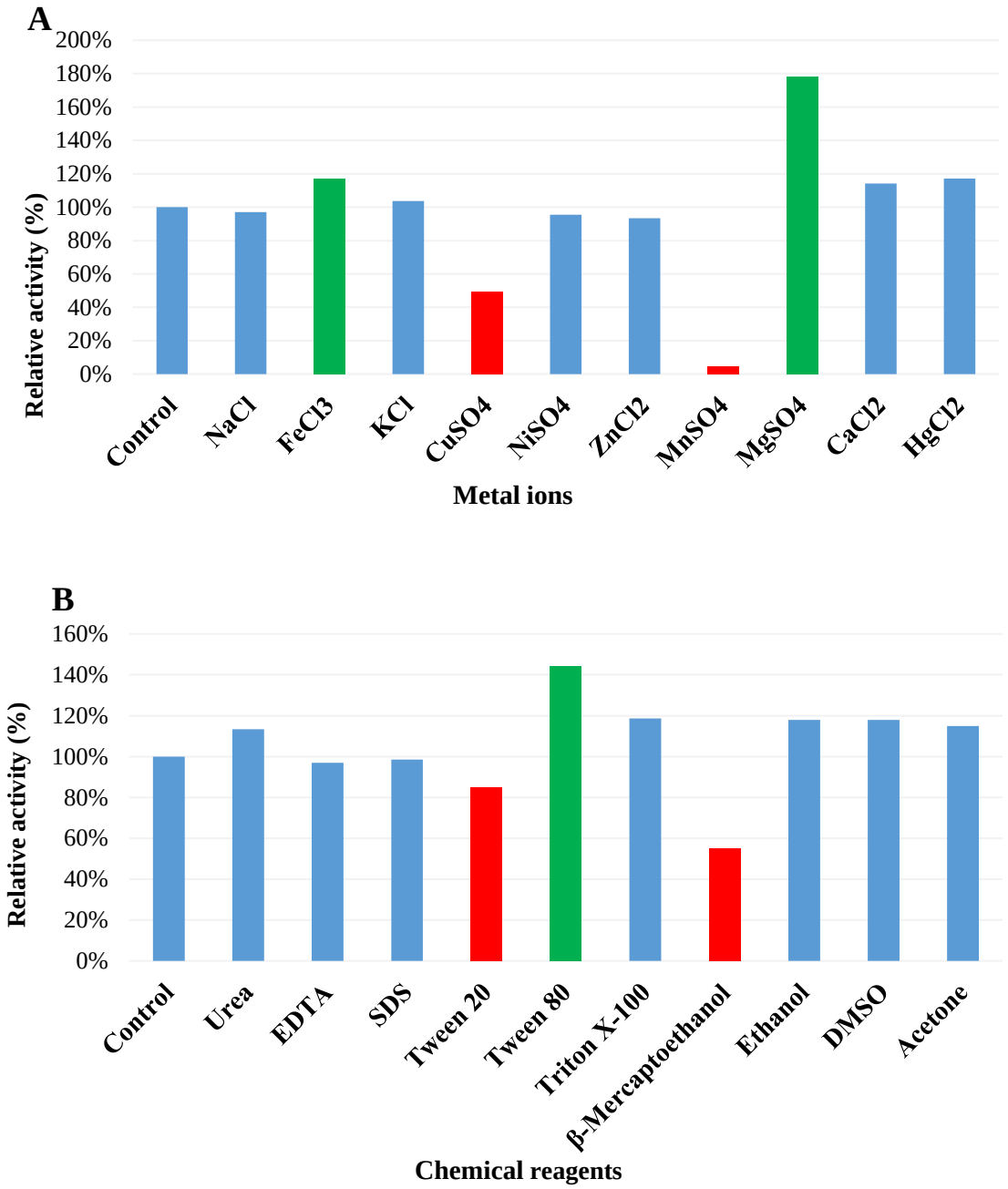

Figure 33: Effect of different metals and chemical reagents on *G. candidum* PO27 α-amylase activity (A: Metals ions ; B:Chemical reagents).

13. Application of purified α-amylase as a desizing agent

In the textile industry, starch is a hydrophilic polymer. Coating it with various sizing agents, however, produces a hydrophobic film that reduces the cotton fabric's absorbency (Aggarwal *et al.*, 2019). It is used to give strength, and prevent thread loss in fabrics including "Jean", which improve the finished product's rigidity.

Enzymatic desizing of cotton with amylases has been at the cutting edge of technology for several decades. It effectively removes starch without damaging the fabric. This process increases the uniformity of wet treatment (Hailemichael and Tadesse, 2022).

The effectiveness of PO27 α-amylase in desizing was demonstrated when starchy white cotton fabric pieces were treated with the amylase enzyme, resulting in the removal of starch, as clearly shown in figure 34 (c). Compared with the control (a), water treatment (b) and commercial enzyme treatment of *Aspergillus oryzae* (d), the enzyme treatment from *G. candidum* PO27 led to significant starch removal.

Research revealed that different sources, including extracellular α-amylase, have been produced by bacteria such as *Bacillus tequilensis* TB5 (Gupta *et al.*, 2024), *Bacillus amyloliquefaciens* (Abd-Elhalim *et al.*, 2023), Fungi and yeasts such as *Trichoderma reesei* (Kalia *et al.*, 2021), *Aspergillus oryzae* (Ahmed and Kolisis, 2011) and *Rhizopus oryzae* (Fukuda *et al.*, 2008), which have been proven highly efficient in the textile industry, giving greatest desizing efficiency.

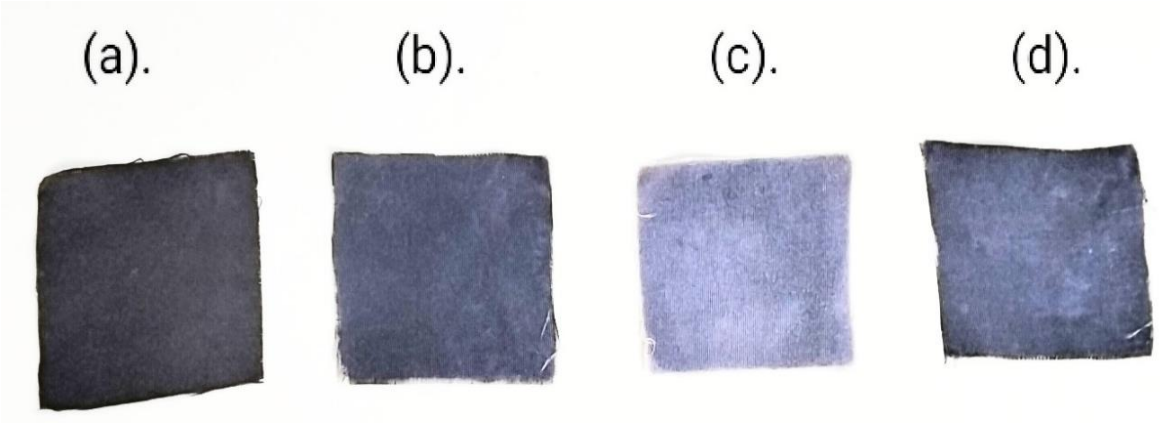

Figure 34: Desizing capability of PO27 α-amylase.

General Conclusion

General conclusion & Outlook

This study successfully achieved its predefined objectives. A collection of 100 yeast strains isolated from diverse biotope in Algeria, such as olive pomace, olive forest soil, thermal water, olive rinse water, mastic oil cake, and vegetable smen has been established, enhancing the laboratory's mycological collection with a range of strains exhibiting biotechnologically relevant metabolic specificities.

Among these isolates, the plate-test-agar method was used to screen enzyme-producing yeasts. The PO27 yeast strain isolated from olive pomace was selected for its ability of high enzyme production, including alpha-amylase, lipase, cellulase and esterase. This isolate was identified as *G. candidum* according to morphological characteristics and was confirmed by 26S rRNA gene sequencing assigned an accession number PP024529 in GenBank.

The second objective of this work is the valorization of olive pomace by-products transformed by fermentation in order to produce a high value-added substance such as alpha amylase of industrial interest. The findings of this study clearly demonstrate that *G. candidum* PO27, isolated from olive pomace, exhibits a significant capability for α-amylase production by utilizing various agro-industrial wastes as natural substrates. Olive pomace emerged as the most effective and cost-efficient source for α-amylase production, with the highest activity achieved through solid-state fermentation (SSF) compared to submerged fermentation (SmF). Amylolytic enzyme-producing yeasts can valorize this by-product, originating from oil mills and causing environmental pollution.

Furthermore, olive pomace waste is selected as the initial raw material due to its substantial annual production and the imperative for processing industries to explore alternative disposal methods. This process harnesses all the organic matter present in these agro-industrial wastes, which are abundant and currently underutilized for this purpose.

Moreover, we present a rational design framework for optimizing the production of the secreted enzyme amylase, from the amylolytic isolate *G. candidum* PO27. The integration of PBD and RSM through CCD was employed to maximize enzyme production. A small set of initial experiments informed the modelling schemes using OFAT approach, and various critical factors were identified and experimentally investigated. Ultimately, the enzyme production was enhanced by a factor of 2.27 when compared to non-optimized environmental conditions.

Optimum fermentation conditions were determined: moisture content 24.77%, malt extract 1.84 g % and $CaCl_2$ 1.84 g %.

The amylolytic activity achieved under optimal conditions for α-amylase production is 412.94 U/g. This closely aligns with the production model, which estimated α-amylase at 421.32 U/g. Under non-optimized conditions, the highest recorded α-amylase activity reached 181.61 U/g.

The maximum production of the α-amylase of interest by *G. candidum* PO27 is achieved after 40 hours and steadily decreases up to 100 hours. During the production phase, the pH undergoes a gradual change and reaches an optimum at pH 5.53. This observation identifies the acidophilic nature of the *G. candidum* PO27 strain, indicating its ability to thrive across a broad pH range.

To facilitate potential enzyme applications, it was separated using various conventional purification methods, including ultrafiltration with a Stirred cell and gel filtration chromatography utilizing the Superdex 75 column. The chromatographic profile on Superdex 75 indicates amylolytic activity in the final peak (peak 5). The enzyme is purified with a purification rate of 6.732 and yields of 9.117%. The enzyme molecular weight was reported as 19.2 KDA using SDS-PAGE.

The glycosylation content indicated the glycoprotein nature of purified *G. candidum* PO29 α-amylase.

Biochemical properties were also studied: purified α-amylase from *G. candidum* PO27 has optimum activity at 70°C and an optimum pH of 5. These two parameters are favorable to the use of this enzyme in several industrial sectors. The thermostability of this enzyme was studied, showing high stability. Thermal treatments lasting 3 h at 70°C and 100°C respectively showed that the α-amylase maintained 58% of its initial activity after 60 min of incubation at 100°C and 54.78 % at 70°C. However, a decrease of about 80.7 % of its activity after 180 min of incubation at 100°C and about 81.3% at 70°C was registered.

The kinetic parameters of the enzyme are determined: the respective K_m at 70°C is 0.114 mg/ml, V_{max} is 588.23 U/ml.

α-Amylase derived from *G. candidum* PO27 strain was shown to have critical characteristics such as high potency and resistance at low pH and high temperatures, as well as good stability to metal ions, surfactants and organic solvents. In this way, the effectiveness of

G. candidum PO27 α-amylase in removing starch stains is remarkable. At the conclusion of this study, we envision the following perspectives:

- Others applications of the enzyme based on the presented characteristics.
- Elucidation of the thermostability and functionality at low pH values of enzyme using molecular tools and proteomic techniques.
- Strain PO27 has shown great enzymatic potential, so it would be interesting to investigate other eventual enzymes for industrial use.
- It would be interesting to study the enzyme's three-dimensional structure by crystallography.
- In-depth study of certain strains from our isolates, given their performance, which closely matches that of the strain in this study.

References

References

Abd-Elhalim BT, Gamal RF, El-Sayed SM, and Abu-Hussien SH (2023). Optimizing alpha-amylase from *Bacillus amyloliquefaciens* on bread waste for effective industrial wastewater treatment and textile desizing through response surface methodology. *Scientific Reports*, *13*(1), 19216. https://doi.org/10.1038/s41598-023-46384-6

Abdel-Hameed S, Ahmed S, Mostafa F, Almasarawi Ola N, and Abdel Wahab W (2022). Preparation and characterization of sugilite glass from basalt for α-amylase immobilization, statistical optimization of the immobilization process and description of free and immobilized enzyme. *Heliyon*, *8*, e09960. https://doi.org/10.1016/j.heliyon.2022.e09960

Abdel-Mageed HM, Barakat AZ, Bassuiny RI, Elsayed AM, Salah HA, Abdel-Aty AM, and Mohamed SA (2022). Biotechnology approach using watermelon rind for optimization of α-amylase enzyme production from *Trichoderma virens* using response surface methodology under solid-state fermentation. *Folia Microbiologica*, *67*(2), 253–264. https://doi.org/10.1007/s12223-021-00929-2

Abdel-Rahman MA, Hassan SED, El-Din MN, Azab MS, El-Belely EF, Alrefaey HMA, and Elsakhawy T (2020). One-factor-at-a-time and response surface statistical designs for improved lactic acid production from beet molasses by *Enterococcus hirae* ds10. *SN Applied Sciences*, *2*(4), 573. https://doi.org/10.1007/s42452-020-2351-x

Abo-Kamer AM, Abd-El-salam IS, Mostafa FA, Mustafa AERA, and Al-Madboly LA (2023). A promising microbial α-amylase production, and purification from *Bacillus cereus* and its assessment as antibiofilm agent against *Pseudomonas aeruginosa* pathogen. *Microbial Cell Factories*, *22*(1), 141. https://doi.org/10.1186/s12934-023-02139-6

Acer Ö, Bekler FM, Pirinççioğlu H, Güven RG, and Güven K (2016). Purification and characterization of thermostable and detergent-stable α-amylase from *Anoxybacillus* sp.

AH1. *Food Technology and Biotechnology*, *54*(1), 70–77. https://doi.org/10.17133/ftb.54.01.16.4122

ù F, and Mobini-Dehkordi M (2012). Application of alpha-amylase in biotechnology. *Journal of Biology and today's world*, 1(1), 39-5039.

Aggarwal R, Dutta T, and Sheikh J (2019). Extraction of amylase from the microorganism isolated from textile mill effluent vis a vis desizing of cotton. *Sustainable Chemistry and Pharmacy*, *14*, 100178. https://doi.org/10.1016/j.scp.2019.100178

Agrawal D, Patidar P, Banerjee T, and Patil S (2004). Production of alkaline protease by *Penicillium* sp. Under SSF conditions and its application to soy protein hydrolysis. *Process Biochemistry*, *39*(8), 977–981. https://doi.org/10.1016/S0032-9592(03)00212-7

Ahmed A (2020). Characterization of pectinase from *Geotrichum candidum* AA15 and its potential application in orange juice clarification. *Journal of King Saud University - Science*, *32*. https://doi.org/10.1016/j.jksus.2019.07.002

Ahmed A, Ejaz U, and Sohail M (2020). Pectinase production from immobilized and free cells of *Geotrichum candidum* AA15 in galacturonic acid and sugars containing medium. *Journal of King Saud University - Science*, *32*(1), 952–954. https://doi.org/10.1016/j.jksus.2019.07.003

Ahmed HE, and Kolisis FN (2011). An investigation into the removal of starch paste adhesives from historical textiles by using the enzyme α-amylase. *Journal of Cultural Heritage*, *12*(2), 169–179. https://doi.org/10.1016/j.culher.2010.08.001

Ahmed SA, Abdella MAA, El-Sherbiny GM, Ibrahim AM, El-Shamy AR, and Atalla SMM (2019). Application of one –factor- at-a-time and statistical designs to enhance α-amylase production by a newly isolate *Bacillus subtilis* strain-MK1. *Biocatalysis and Agricultural Biotechnology*, *22*, 101397. https://doi.org/10.1016/j.bcab.2019.101397

Ahmed SA, Mostafa FA, Helmy WA, and Abdel-Naby MA (2017). Improvement of bacterial α-amylase production and application using two steps statistical factorial design. *Biocatalysis and Agricultural Biotechnology*, *10*, 224–233. https://doi.org/10.1016/j.bcab.2017.03.004

Ait Kaki - El-Hadef El-Okki A, Gagaoua M, Bourekoua H, Kahina H, Bennamoun L, Djekrif-Dakhmouche S, El Hadef El Okki M, and Zahia M (2017). Improving bread quality with the application of a newly purified thermostable α-amylase from *Rhizopus oryzae* FSIS4. *Foods*, *6*, 1. https://doi.org/10.3390/foods6010001

Akcan N, Uyar F, and Güven A (2009). Submerged Kültürü ile *Bacillus subtilis* RSKK96'dan α-Amilaz Üretimi. *Kafkas Universitesi Veteriner Fakultesi Dergisi*. https://doi.org/10.9775/kvfd.2010.3036

Alanio A (2013). La spectrométrie de masse de type MALDI-TOF en mycologie clinique: Avantages réels, écueils potentiels. *Journal des Anti-infectieux*, *15*(2), 71–82. https://doi.org/10.1016/j.antinf.2013.04.001

Al-Dhabi NA, Esmail GA, Ghilan AKM, Arasu MV, Duraipandiyan V, and Ponmurugan K (2020). Isolation and purification of starch hydrolysing amylase from *Streptomyces* sp. Al-Dhabi-46 obtained from the Jazan region of Saudi Arabia with industrial applications. *Journal of King Saud University - Science*, *32*(1), 1226–1232. https://doi.org/10.1016/j.jksus.2019.11.018

Almanaa TN, Vijayaraghavan P, Alharbi NS, Kadaikunnan S, Khaled JM, and Alyahya SA (2020). Solid state fermentation of amylase production from *Bacillus subtilis* D19 using agro-residues. *Journal of King Saud University - Science*, *32*(2), 1555–1561. https://doi.org/10.1016/j.jksus.2019.12.011

Alonazi M, Karray A, Badjah-Hadj-Ahmed AY, and Ben Bacha A (2021). Alpha amylase from *Bacillus pacificus* associated with brown algae *Turbinaria ornata*: Cultural conditions,

purification, and biochemical characterization. *Processes*, *9*(1), Article 1. https://doi.org/10.3390/pr9010016

Alper I, Frenette M, and Labrie S (2011). Ribosomal DNA polymorphisms in the yeast *Geotrichum candidum*. *Fungal Biology*, *115*(12), 1259–1269. https://doi.org/10.1016/j.funbio.2011.09.002

Ambehabati KK, Hanapi SZ, Baz AFE, Sayyed RZ, Dailin DJ, and Enshasy HAE (2020). Isolation and identification studies on potential xylanase producing strain *Trichoderma* sp. WICC F46 isolated from tropical soil. *Journal of Scientific and Industrial Research*, *79*(2). https://doi.org/10.56042/jsir.v79i2.68471

Arif AB, Sasmitaloka KS, Winarti C, and Wahyudiono (2019). Effect of liquefaction time and enzyme addition on liquid sugar production from sweet sorghum starch by enzymatic hydrolysis. *IOP Conference Series: Earth and Environmental Science*, *250*, 012042. https://doi.org/10.1088/1755-1315/250/1/012042

Arman Z, Sondana GA, Fikriyyah NN, Afifah ZN, Balqis M, Hasanah R, Risandi A, Sofiana I, Nisa H, Ridawati Muktiningsih M, and Sukmawati D (2020). Screening of amylolytic and cellulolytic yeast from *Dendrobium spathilingue* in Bali Botanical Garden, Indonesia. 050013. https://doi.org/10.1063/5.0007802

Arunachallam P, Kumaravel V, and Gopal SR (2023). Purification and biochemical characterization of α- amylase from *Aspergillus tamarii* MTCC5152. *Preparative Biochemistry and Biotechnology*, *0*(0), 1–10. https://doi.org/10.1080/10826068.2023.2235694

Arunsasi M, Kani SM, Jegadeesh G, and Ravikumar M (2010). Submerged fermentation of amylase enzyme by *Aspergillus flavus* using *Cocos nucifera* meal. *Kathmandu University Journal of Science, Engineering and Technology*, *6*(2), Article 2. https://doi.org/10.3126/kuset.v6i2.4015

Attanayaka DPSTG, De Silva SNT, Nirosha SF, and Aththanayaka AMWS (2009). Isolation of raw starch hydrolysing fungi and purification of α-amylase from *Geotrichum candidum* CMSS06. *Journal of the National Science Foundation of Sri Lanka*, *37*(2), 93. https://doi.org/10.4038/jnsfsr.v37i2.1064

Baffi MA, dos Santos Bezerra C, Arévalo-Villena M, Isabel Briones-Pérez A, Gomes E, and Da Silva R (2011). Isolation and molecular identification of wine yeasts from a Brazilian vineyard. *Annals of Microbiology*, *61*(1), Article 1. https://doi.org/10.1007/s13213-010-0099-z

Bagheri A, Khodarahmi R, and Mostafaie A (2014). Purification and biochemical characterisation of glucoamylase from a newly isolated *Aspergillus niger*: Relation to starch processing. *Food Chemistry*, *161*, 270–278. https://doi.org/10.1016/j.foodchem.2014.03.095

Balakrishnan M, Jeevarathinam G, Kumar SKS, Muniraj I, and Uthandi S (2021). Optimization and scale-up of α-amylase production by *Aspergillus oryzae* using solid-state fermentation of edible oil cakes. *BMC Biotechnology*, *21*(1), 33. https://doi.org/10.1186/s12896-021-00686-7

Baltas N, Dincer B, Ekinci AP, Kolayli S, and Adiguzel A (2016). Purification and characterization of extracellular α-amylase from a thermophilic *Anoxybacillus thermarum* A4 strain. *Brazilian Archives of Biology and Technology*, *59*, e16160346. https://doi.org/10.1590/1678-4324-2016160346

Belén Flórez A, Álvarez-Martín P, López-Díaz TM, and Mayo B (2007). Morphotypic and molecular identification of filamentous fungi from spanish blue-veined cabrales cheese, and typing of *Penicillium roqueforti* and *Geotrichum candidum* isolates. *International Dairy Journal*, *17*(4), 350–357. https://doi.org/10.1016/j.idairyj.2006.04.002

Benabda O, M'hir S, Kasmi M, Mnif W, and Hamdi M (2019). Optimization of protease and amylase production by *Rhizopus oryzae* cultivated on bread waste using solid-state fermentation. *Journal of Chemistry*, *2019*, 3738181. https://doi.org/10.1155/2019/3738181

Bergman LW (2001). Growth and maintenance of yeast. In PN MacDonald (Ed.), *Two-Hybrid Systems: Methods and Protocols* (pp. 9–14). Humana Press. https://doi.org/10.1385/1-59259-210-4:009

Bernfeld P (1955). Amylases, α and β. In *Methods in Enzymology* (Vol. 1, pp. 149–158). Academic Press. https://doi.org/10.1016/0076-6879(55)01021-5

Bertolini MC, Laramée L, Thomas DY, Cygler M, Schrag JD, and Vernet T (1994). Polymorphism in the lipase genes of *Geotrichum candidum* strains. *European Journal of Biochemistry*, *219*(1–2), 119–125. https://doi.org/10.1111/j.1432-1033.1994.tb19921.x

Bharathi D, and Rajalakshmi G (2019). Microbial lipases: An overview of screening, production and purification. *Biocatalysis and Agricultural Biotechnology*, *22*, 101368. https://doi.org/10.1016/j.bcab.2019.101368

Bijttebier A, Goesaert H, and Delcour J (2008). Amylase action pattern on starch polymers. *Biologia*, *63*, 989–999. https://doi.org/10.2478/s11756-008-0169-x

Boutrou R, and Guéguen M (2005). Interests in *Geotrichum candidum* for cheese technology. *International Journal of Food Microbiology*, *102*(1), 1–20. https://doi.org/10.1016/j.ijfoodmicro.2004.12.028

Boutrou R, Kerriou L, and Gassi JY (2006). Contribution of *Geotrichum candidum* to the proteolysis of soft cheese. *International Dairy Journal*, *16*(7), 775–783. https://doi.org/10.1016/j.idairyj.2005.07.007

Brabcová J, Demianová Z, Vondrášek J, Jágr M, Zarevúcka M, and Palomo JM (2013). Highly selective purification of three lipases from *Geotrichum candidum* 4013 and their characterization and biotechnological applications. *Journal of Molecular Catalysis B: Enzymatic*, *98*, 62–72. https://doi.org/10.1016/j.molcatb.2013.09.012

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254. https://doi.org/10.1006/abio.1976.9999

Burhanoğlu T, Sürmeli Y, and Şanlı-Mohamed G (2020). Identification and characterization of novel thermostable *α*-amylase from *Geobacillus* sp. GS33. *International Journal of Biological Macromolecules*, *164*, 578–585. https://doi.org/10.1016/j.ijbiomac.2020.07.171

Burkert JFDM, Maldonado RR, Filho FM, and Rodrigues MI (2005). Comparison of lipase production by *Geotrichum candidum* in stirring and airlift fermenters. *Journal of Chemical Technology and Biotechnology*, *80*(1), 61–67. https://doi.org/10.1002/jctb.1157

Butler EE, and Petersen LJ (1972). Endomyces geotrichum a Perfect State of *Geotrichum candidum*. *Mycologia*, *64*(2), 365–374. https://doi.org/10.2307/3757839

Buzzini P, and Martini A (2002). Extracellular enzymatic activity profiles in yeast and yeast-like strains isolated from tropical environments. *Journal of Applied Microbiology*, *93*(6), 1020–1025. https://doi.org/10.1046/j.1365-2672.2002.01783.x

Buzzini P, Turchetti B, and Yurkov A (2018). Extremophilic yeasts: The toughest yeasts around? *Yeast*, *35*(8), 487–497. https://doi.org/10.1002/yea.3314

Cardoso SM, Silva AMS, and Coimbra MA (2002). Structural characterisation of the olive pomace pectic polysaccharide arabinan side chains. *Carbohydrate Research*, *337*(10), 917–924. https://doi.org/10.1016/S0008-6215(02)00082-4

Carrim AJI, Barbosa EC, and Vieira JDG (2006). Enzymatic activity of endophytic bacterial isolates of Jacaranda decurrens Cham. (Carobinha-do-campo). *Brazilian Archives of Biology and Technology*, *49*(3), 353–359. https://doi.org/10.1590/S1516-89132006000400001

Chaib I, Dakhmouche-Djekrif S, Bennamoun L, Nouadri T (2024). Extracellular enzymes producing yeasts study: Cost-effective production of α-amylase by a newly isolated thermophilic yeast *Geotrichum candidum* PO27. *AIMS Microbiology*, *10*(1), https://doi.org/10.3934/microbiol.2024006

Chakraborty S, Khopade A, Kokare C, Mahadik K, and Chopade B (2009). Isolation and characterization of novel α-amylase from marine *Streptomyces* sp. D1. *Journal of Molecular Catalysis B: Enzymatic*, *58*(1), 17–23. https://doi.org/10.1016/j.molcatb.2008.10.011

Chávez-Camarillo GMa, Lopez-Nuñez PV, Jiménez-Nava RA, Aranda-García E, and Cristiani-Urbina E (2022). Production of extracellular α-amylase by single-stage steady-state continuous cultures of *Candida wangnamkhiaoensis* in an airlift bioreactor. *PLoS ONE*, *17*(3), e0264734. https://doi.org/10.1371/journal.pone.0264734

Coman G, Leuştean I, Georgescu L, and Bahrim G (2012). Optimization of protein production by *Geotrichum candidum* MIUG 2.15 by cultivation on paper residues, using response surface methodology. *Bioresources*, *7*, 5290–5303. https://doi.org/10.15376/biores.7.4.5290-5303

Cordeiro CAM, Martins MLL, and Luciano AB (2002). Production and properties of alpha-amylase from thermophilic *Bacillus* sp. *Brazilian Journal of Microbiology*, *33*, 57–61. https://doi.org/10.1590/S1517-83822002000100012

Costa-Silva TA, Carvalho AKF, Souza CRF, De Castro HF, Bachmann L, Said S, and Oliveira WP (2021). Enhancement lipase activity via immobilization onto chitosan beads used

as seed particles during fluidized bed drying: Application in butyl butyrate production. *Applied Catalysis A: General*, *622*, 118217. https://doi.org/10.1016/j.apcata.2021.118217

Dakhmouche Djekrif S, Bennamoun L, Labbani FZK, Ait Kaki A, Nouadri, T, Pauss A, Meraihi Z, and Gillmann L (2021). An alkalothermophilic amylopullulanase from the yeast *Clavispora lusitaniae* abs7: Purification, characterization and potential application in laundry detergent. *Catalysts*, *11*(12), 1438. https://doi.org/10.3390/catal11121438

De Mot R, and Verachtert H (1986). Secretion of alpha-amylase and multiple forms of glucoamylase by the yeast *Trichosporon pullulans*. *Canadian Journal of Microbiology*, *32*(1), 47–51. https://doi.org/10.1139/m86-009

Deak T (2006). Environmental factors influencing yeasts. In G. Péter and C. Rosa (Eds.), *Biodiversity and Ecophysiology of Yeasts* (pp. 155–174). Springer. https://doi.org/10.1007/3-540-30985-3_8

Deák T, and Péter G (2013). Developments in yeast taxonomy. *Acta Alimentaria*, *42*(1), 55–68. https://doi.org/10.1556/aalim.42.2013.1.6

Dickmanns A, Ballschmiter M, Liebl W, and Ficner R (2006). Structure of the novel α-amylase AmyC from *Thermotoga maritima*. *Acta Crystallographica Section D: Biological Crystallography*, *62*(3), 262–270. https://doi.org/10.1107/S0907444905041363

Divya K, and Padma PN (2014). Yeast isolates from diverse sources for cold- active polygalacturonase and amylase production. *3*(4), 5.

Djekrif-Dakhmouche S (2016). Production and characterization of amylopullulanase from the yeast *Clavispora lusitaniae* abs7 isolated from wheat, grown and stored in arid zones, doctoral thesis. *Compiegne University of Technology, (UTS),* Compiegne France 211.

Djekrif-Dakhmouche S, Gheribi-Aoulmi Z, Meraihi Z, and Bennamoun L (2006). Application of a statistical design to the optimization of culture medium for α-amylase production

by *Aspergillus niger* ATCC 16404 grown on orange waste powder. *Journal of Food Engineering*, *73*(2), 190–197. https://doi.org/10.1016/j.jfoodeng.2005.01.021

Djekrif-Dakhmouche S, Gillmann L, Bennamoun L, Ait Kaki - El-Hadef El-Okki A, Labbani K, Nouadri T, and Zahia M (2016). Amylolytic yeasts: producers of α-amylase and pullulanase. *International Journal of Life-Sciences Scientific Research*.

Dubois Michel, Gilles KA, Hamilton JK, Rebers PA, and Smith Fred (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356. https://doi.org/10.1021/ac60111a017

Effiom H, and Lennox J (2022). Optimization of independent variables for the production of extracellular alpha amylase by *Bacillus subtilis* IMD34 using Plackett-Burman Design. *Sultan Qaboos University Journal for Science [SQUJS]*, *27*(2), Article 2. https://doi.org/10.53539/squjs.vol27iss2pp77-83

Egbune EO, Avwioroko OJ, Anigboro AA, Aganbi E, Amata AI, and Tonukari NJ (2022). Characterization of a surfactant-stable α-amylase produced by solid-state fermentation of cassava (*Manihot esculenta* Crantz) tubers using *Rhizopus oligosporus*: Kinetics, thermal inactivation thermodynamics and potential application in laundry industries. *Biocatalysis and Agricultural Biotechnology*, *39*, 102290. https://doi.org/10.1016/j.bcab.2022.102290

Ekpenyong MG, Antai SP, Asitok AD, and Ekpo BO (2017). Plackett-Burman Design and Response surface optimization of medium trace nutrients for glycolipopeptide biosurfactant production. *Iranian Biomedical Journal*, *21*(4), 249–260. https://doi.org/10.18869/acadpub.ibj.21.4.249

El-Enshasy HA, Abdel Fattah YR, and Othman NZ (2013). Amylases: Characteristics, sources, production, and applications. In S. Yang, H. A. El-Enshasy, and N. Thongchul (Eds.), *Bioprocessing Technologies in Biorefinery for Sustainable Production of Fuels,*

Chemicals, and Polymers (1st ed., pp. 111–130). Wiley. https://doi.org/10.1002/9781118642047.ch7

Eliskases-Lechner F (2002). *Geotrichum candidum*. In H. Roginski (Ed.), *Encyclopedia of Dairy Sciences* (pp. 1229–1234). Elsevier. https://doi.org/10.1016/B0-12-227235-8/00184-X

Eliskases-Lechner F, Guéguen M, and Panoff JM (2011). Yeasts and Molds | *Geotrichum candidum*. In *Encyclopedia of Dairy Sciences* (pp. 765–771). Elsevier. https://doi.org/10.1016/B978-0-12-374407-4.00365-4

Elmansy EA, Asker MS, El-Kady EM, Hassanein SM, and El-Beih FM (2018). Production and optimization of α-amylase from thermo-halophilic bacteria isolated from different local marine environments. *Bulletin of the National Research Centre*, *42*(1), 31. https://doi.org/10.1186/s42269-018-0033-2

Ensari NY, Otludil B, and Aytekin MC (1995). Effect of starch induced bacterial growth and amylase production in *Bacillus subtilis*. *Starch - Stärke*, *47*(8), 315–321. https://doi.org/10.1002/star.19950470807

Erfanimoghadam MR, and Homaei A (2023). Identification of new amylolytic enzymes from marine symbiotic bacteria of *Bacillus* Species. *Catalysts*, *13*(1), Article 1. https://doi.org/10.3390/catal13010183

Ertan F, Balkan B, Balkan S, and Aktac T (2006). Solid state fermentation for the production of α-amylase from *Penicillium chrysogenum* using mixed agricultural by-products as substrate. *Biologia*, *61*(6), 657–661. https://doi.org/10.2478/s11756-006-0137-2

Falih AM (1998). Effect of heavy-metals on amylolytic activity of the soil yeasts *Geotrichum capitatum* and *Geotrichum candidum*. *Bioresource Technology*, *66*(3), 213–217. https://doi.org/10.1016/S0960-8524(98)00063-7

Farooq MA, Ali S, Hassan A, Tahir HM, Mumtaz S, and Mumtaz S (2021). Biosynthesis and industrial applications of α-amylase: A review. *Archives of Microbiology*, *203*(4), 1281–1292. https://doi.org/10.1007/s00203-020-02128-y

Featherstone S (2015). 8—Cleaning and sanitising. In S. Featherstone (Ed.), *A Complete Course in Canning and Related Processes (Fourteenth Edition)* (pp. 149–171). Woodhead Publishing. https://doi.org/10.1016/B978-0-85709-677-7.00008-6

Febriani Rayyana Ulya M, Oesman F, Akhmaloka, and Iqbalsyah TM (2019). Low molecular weight alkaline thermostable α-amylase from *Geobacillus* sp. Nov. *Heliyon*, *5*(7). https://doi.org/10.1016/j.heliyon.2019.e02171

Fincan, SA, Özdemir S, Karakaya A, Enez B, Mustafov SD, Ulutaş MS, and Şen F (2021). Purification and characterization of thermostable α-amylase produced from *Bacillus licheniformis* So-B3 and its potential in hydrolyzing raw starch. *Life Sciences*, *264*, 118639. https://doi.org/10.1016/j.lfs.2020.118639

Finore I, Di Donato P, Poli A, Kirdar B, Kasavi C, Toksoy E, Nicolaus B, and Lama L (2014). Use of agro waste biomass for α-amylase production by *Anoxybacillus amylolyticus:* Purification and properties. *6*, 320–3266.

Finore I, Kasavi C, Poli A, Romano I, Toksoy Oner E, Kirdar B, Dipasquale L, Nicolaus B, and Lama L (2011). Purification, biochemical characterization and gene sequencing of a thermostable raw starch digesting α-amylase from *Geobacillus thermoleovorans* subsp. *Stromboliensis* subsp. Nov. *World Journal of Microbiology and Biotechnology*, *27*, 2425–2433. https://doi.org/10.1007/s11274-011-0715-5

Fossi BT, Tavea F,and Ndjouenkeu R (2005). Production and partial characterization of a thermostable amylase from ascomycetes yeast strain isolated from starchy soils. *African Journal of Biotechnology* , 4 (1), 14-18.

Fukuda T, Kato-Murai M, Kuroda K, Ueda M, and Suye S (2008). Improvement in enzymatic desizing of starched cotton cloth using yeast codisplaying glucoamylase and cellulose-binding domain. *Applied Microbiology and Biotechnology*, *77*(6), 1225–1232. https://doi.org/10.1007/s00253-007-1263-7

Gad AM, Suleiman WB, El-Sheikh HH, Elmezayen HA, and Beltagy EA (2022). Characterization of cellulase from *Geotrichum candidum* Strain Gad1 approaching bioethanol production. *Arabian Journal for Science and Engineering*, *47*(6), 6837–6850. https://doi.org/10.1007/s13369-021-06391-z

Gangadharan D, Sivaramakrishnan S, Nampoothiri KM, Sukumaran RK, and Pandey A. (2008). Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*. *Bioresource Technology*, *99*(11), 4597–4602. https://doi.org/10.1016/j.biortech.2007.07.028

Gente S, Sohier D, Coton E, Duhamel C, and Gueguen M (2006). Identification of *Geotrichum candidum* at the species and strain level: Proposal for a standardized protocol. *Journal of Industrial Microbiology and Biotechnology*, *33*(12), 1019–1031. https://doi.org/10.1007/s10295-006-0130-3

Gopinath SCB, Anbu P, Arshad MKM, Lakshmipriya T, Voon CH, Hashim U, and Chinni S V. (2017). Biotechnological processes in microbial amylase production. *BioMed Research International*, *2017*, 1–9. https://doi.org/10.1155/2017/1272193

Goswami GK, and Rawat S (2015). Microbial xylanase and their applications —A review. *International Journal of Current Research and Academic Review* , 3(6), 435-450

Gueguen M, and Jacquet J (1982). Etudes sur les caractères culturaux et la morphologie de *Geotrichum candidum* Link. *Le Lait*, *62* (621–622), 625–644. https://doi.org/10.1051/lait:1982621-62241

Gueguen M, and Lenoir J (1976). Caractères du système protéolytique de *Geotrichum candidum*. *Le Lait*, *56*(557), 439–448. https://doi.org/10.1051/lait:197655723

Gunam IBW, Sujana IGA, Wijaya IMM, Setiyo Y, Putra IWWP, and Suriati L (2021). Isolation and selection of amylase-producing microbes isolated from ragi tape and cassava tape available on the markets. *IOP Conference Series: Earth and Environmental Science*, *913*(1), 012041. https://doi.org/10.1088/1755-1315/913/1/012041

Gupta N, Paul JS, and Jadhav SK (2024). Biovalorizing agro-waste 'de-oiled rice bran' for thermostable, alkalophilic and detergent stable α-amylase production with its application as laundry detergent additive and textile desizer. *International Journal of Biological Macromolecules*, *256*, 128470. https://doi.org/10.1016/j.ijbiomac.2023.128470

Gupta R, Gigras P, Mohapatra H, Goswami VK, and Chauhan B (2003). Microbial α-amylases: A biotechnological perspective. *Process Biochemistry*, *38*(11), 1599–1616. https://doi.org/10.1016/S0032-9592(03)00053-0

Hailemichael F, and Tadesse S (2022). Process Optimization and Industrial Application of Microbial Amylases: A Review, *Global Scientific Journals* ,*10* (4).

Hamilton LM, Kelly CT, and Fogarty WM (1999). Purification and properties of the raw starch-degrading α- amylase of *Bacillus* sp. IMD 434. *Biotechnology Letters*, *21*(2), 111–115. https://doi.org/10.1023/A:1005413816101

Hattingh M, Alexander A, Meijering I, van Reenen CA, and Dicks LMT (2014). Malting of barley with combinations of *Lactobacillus plantarum*, *Aspergillus niger*, *Trichoderma* reesei, *Rhizopus oligosporus* and *Geotrichum candidum* to enhance malt quality. *International Journal of Food Microbiology*, *173*, 36–40. https://doi.org/10.1016/j.ijfoodmicro.2013.12.017

Hlavsová K, Zarevúcka M, Wimmer Z, Macková M, and Sovová H (2009). *Geotrichum candidum* 4013: Extracellular lipase versus cell-bound lipase from the single strain. *Journal of Molecular Catalysis B: Enzymatic*, *61*(3), 188–193. https://doi.org/10.1016/j.molcatb.2009.06.012

Hollmann F, Arends IWCE, Buehler K, Schallmey A, and Bühler B (2011). Enzyme-mediated oxidations for the chemist. *Green Chemistry*, *13*(2), 226–265. https://doi.org/10.1039/C0GC00595A

Hoog S, and Smith M (2004). Ribosomal gene phylogeny and species delimitation in *Geotrichum* and its teleomorph. *Studies in Mycology*, *50*, 489–515.

Hudecová A, and Liptáková D (2009). *Influence of temperature on the surface growth of Geotrichum candidum*. *Acta Chimica Slovaca*, *2* (2),75 - 87

Humbird D, and Fei Q (2016). Chapter 20—Scale-up considerations for biofuels. In C. A. Eckert and C. T. Trinh (Eds.), *Biotechnology for Biofuel Production and Optimization* (pp. 513–537). Elsevier. https://doi.org/10.1016/B978-0-444-63475-7.00020-0

Ibrar M, Zuberi A, Amir I, Imran M, and Noor Z (2017). Effect of probiotic *Geotrichum candidum* on early rearing of labeo rohita (Hamilton, 1822). *Turkish Journal of Fisheries and Aquatic Sciences*, *17*(6). https://doi.org/10.4194/1303-2712-v17_6_19

Ichikawa M, Scott DA, Losfeld ME, and Freeze HH (2014). The metabolic origins of mannose in glycoproteins. *The Journal of Biological Chemistry*, *289*(10), 6751–6761. https://doi.org/10.1074/jbc.M113.544064

Jesubunmi C, and Ogbonna J (2022). Production of cellulase and amylase enzymes in both solid and liquid states by two species of fungi. *Asian Journal of Biotechnology and Bioresource Technology*, 1–11. https://doi.org/10.9734/ajb2t/2022/v8i130115

Jha S, Rai H, Chattopadhyay R, Kaur VP, Indupriya M, and Shanti V (2013). Effect of metal ions on amylase production. *Recent Research in Science and Technology*.

Jujjavarapu S, and Dhagat S (2018). Evolutionary trends in industrial production of α-amylase. *Current Biotechnology*, *07*. https://doi.org/10.2174/2211550107666180816093436

Kalia S, Bhattacharya A, Prajapati SK, and Malik A (2021). Utilization of starch effluent from a textile industry as a fungal growth supplement for enhanced α-amylase production for industrial application. *Chemosphere*, *279*, 130554. https://doi.org/10.1016/j.chemosphere.2021.130554

Kamilari E, Stanton C, Reen FJ, and Ross RP (2023). Uncovering the Biotechnological Importance of *Geotrichum candidum*. *Foods*, *12*(6), Article 6. https://doi.org/10.3390/foods12061124

Kammoun R, Naili B, and Bejar S (2008). Application of a statistical design to the optimization of parameters and culture medium for α-amylase production by *Aspergillus oryzae* CBS 819.72 grown on gruel (wheat grinding by-product). *Bioresource Technology*, *99*(13), 5602–5609. https://doi.org/10.1016/j.biortech.2007.10.045

Karam EA, Abdel Wahab WA, Saleh SAA, Hassan ME, Kansoh AL, and Esawy MA (2017). Production, immobilization and thermodynamic studies of free and immobilized *Aspergillus awamori* amylase. *International Journal of Biological Macromolecules*, *102*, 694–703. https://doi.org/10.1016/j.ijbiomac.2017.04.033

Karam NE, and Idoui T (2008). Lactic acid bacteria from Jijel's traditional butter: Isolation, Identification and major technological traits. *Grasas y Aceites*, *59*. https://doi.org/10.3989/gya.2008.v59.i4.530

Kawtharani H, Beaufort S, Anson P, Taillandier P, Mathieu F, and Snini SP (2022). Impact of the inoculation method of *Geotrichum candidum*, Used as biocontrol agent, on T-2 toxin produced by *Fusarium sporotrichioides* and *F. langsethiae* during the malting Process. *Toxins*, *14*(4), 239. https://doi.org/10.3390/toxins14040239

Kawtharani H, Snini SP, Heang S, Bouajila J, Taillandier P, Mathieu F, and Beaufort S (2020). Phenyllactic acid produced by *Geotrichum candidum* reduces *Fusarium sporotrichioides* and *F. langsethiae* growth and T-2 toxin concentration. *Toxins, 12*(4), Article 4. https://doi.org/10.3390/toxins12040209

Kennedy JF (1988). *Carbohydrate chemistry*. Clarendon Press ; Oxford University Press. http://catdir.loc.gov/catdir/enhancements/fy0638/87007655-t.html

Khadka S, Khadka D, Poudel RC, Bhandari M, Baidya P, Sijapati J, and Maharjan J (2022). Production optimization and biochemical characterization of cellulase from *Geobacillus* sp. KP43 isolated from hot spring water of Nepal. *BioMed Research International*, *2022*, 1–12. https://doi.org/10.1155/2022/6840409

Khelil O, Choubane S, Maredj K, Mahiddine FZ, and Hamouta A (2022). UV mutagenesis for the overproduction of thermoalkali-stable α-amylase from *Bacillus subtilis* TLO3 by fermentation of stale bread: Potential application as detergent additive. *Biocatalysis and Agricultural Biotechnology*, *43*, 102403. https://doi.org/10.1016/j.bcab.2022.102403

Khusro A, Kannan B, Aarti C, and Agastian P (2017). Optimization of thermo-alkali stable amylase production and biomass yield from *Bacillus* sp. under submerged cultivation. *Fermentation*, *3*, 7. https://doi.org/10.3390/fermentation3010007

Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*(2), 111–120. https://doi.org/10.1007/BF01731581

Kizhakedathil MPJ, and CSD (2021). Acid stable α-amylase from *Pseudomonas balearica* VITPS19—Production, purification and characterization. *Biotechnology Reports*, *30*, e00603. https://doi.org/10.1016/j.btre.2021.e00603

Koňuchová M, Lehotová V, Šípková A, Piecková E, and Valík (2016). Biodiversity Evaluation of *Geotrichum candidum* Link. Is Arthrosporic Nucleus Number in

Geotrichum candidum Related to the Fungus Biodiversity? *Scientia Agriculturae Bohemica*, *47*, 181–186. https://doi.org/10.1515/sab-2016-0026

Krishnankutty V (2024). Optimization of lipase production by response surface methodology from *Serratia marcescens* VT 1 isolated from oil contaminated soil. *Biologia*. https://doi.org/10.1007/s11756-024-01614-8

Kurtzman CP, Fell JW, and Boekhout T (2011). Chapter 1—definition, classification and nomenclature of the Yeasts. In C. P. Kurtzman, J. W. Fell, and T. Boekhout (Eds.), *The Yeasts (Fifth Edition)* (pp. 3–5). Elsevier. https://doi.org/10.1016/B978-0-444-52149-1.00001-X

Kwon YM, Choi HS, Lim JY, Jang HS, and Chung D (2020). Characterization of amylolytic activity by a marine-derived yeast *Sporidiobolus pararoseus* PH-Gra1. *Mycobiology*, *48*(3), 195–203. https://doi.org/10.1080/12298093.2020.1763100

Ladevèze S, Haon M, Villares A, Cathala B, Grisel S, Herpoël-Gimbert I, Henrissat B, and Berrin, J.-G. (2017). The yeast *Geotrichum candidum* encodes functional lytic polysaccharide monooxygenases. *Biotechnology for Biofuels*, *10*(1), 215. https://doi.org/10.1186/s13068-017-0903-0

Lanka S, and B TT (2018). Screening and isolation of lipase producing fungi from marine water obtained from Machilipatnam Costal region. *International Journal of Pharmacognosy and Phytochemical Research*, *9*(07). https://doi.org/10.25258/phyto.v9i07.11157

Lanka S, and Naveena Lavanya Latha J (2015). A Short review on various screening methods to isolate potential lipase producers: Lipases-the present and future enzymes of biotech industry. *International Journal of Biological Chemistry*, *9*, 207–219. https://doi.org/10.3923/ijbc.2015.207.219

Lee HL, Shin J, Koh CH, Ryu H, Lee DJ, and Sohn C (2002). Surface sizing with cationic starch: Its effect on paper quality and papermaking process. *Tappi Journal*, *1*, 34–40.

Lee YJ, Choi YR, Lee SY, Park JT, Shim JH, Park KH, and Kim JW (2011). Screening wild yeast strains for alcohol fermentation from various fruits. *Mycobiology*, *39*(1), 33–39. https://doi.org/10.4489/MYCO.2011.39.1.033

Leite P, Salgado JM, Venâncio A, Domínguez JM, and Belo I (2016). Ultrasounds pretreatment of olive pomace to improve xylanase and cellulase production by solid-state fermentation. *Bioresource Technology*, *214*, 737–746. https://doi.org/10.1016/j.biortech.2016.05.028

Lineweaver H, and Burk D (1934). The Determination of enzyme dissociation constants. *Journal of the American Chemical Society*, *56*(3), 658–666. https://doi.org/10.1021/ja01318a036

Link JHF. *(1809) 1809:* Observationes in ordines plantarum naturales. *Dissertatio Ima. Gesellschaft Naturforschender Freunde zu Berlin, Magazin 3(1): 3-42.* - *Biota of NZ.* (1809). Retrieved 4 February 2024, from https://biotanz.landcareresearch.co.nz/references/1cb0f202-36b9-11d5-9548-00d0592d548c

Link JHF (1824) *Botrytis geotricha* Link, *Caroli a Linné Species Plantarum exhibentes Plantas Rite Cognitas ad Genera Relata*s , 6 (1): 53 .

Loo J (2006). Identification and characterisation of a locally isolated lipolytic microfungus—*Geotrichum candidum*. *Malaysian Journal of Microbiology*, *2*, 22–29.

Luang-In V, Yotchaisarn M, Saengha W, Udomwong P, Deeseenthum S, and Maneewan K (2019). Isolation and identification of amylase-producing bacteria from soil in Nasinuan Community Forest, Maha Sarakham, Thailand. *Biomedical and Pharmacology Journal*, *12*(3), 1061–1068. https://doi.org/10.13005/bpj/1735

Mahmood S, Shahid M, Irfan M, Nadeem M,and Syed Q (2018). Partial characterization of α-amylase produced from *Aspergillus nige*r using potato peel as substrate. *Punjab University Journal of Zoology*, *33*. https://doi.org/10.17582/pujz/2018.33.1.22.27

Maldonado RR (2017). A review on *Geotrichum* lipases: production, purification, immobilization and applications. *Chemical and Biochemical Engineering Quarterly Journal*, *30*(4), 439–454. https://doi.org/10.15255/CABEQ.2016.907

Marcellino N, Beuvier E, Grappin R, Guéguen M, and Benson DR (2001). Diversity of *Geotrichum candidum* strains isolated from traditional cheesemaking fabrications in France. *Applied and Environmental Microbiology*, *67*(10), 4752–4759. https://doi.org/10.1128/AEM.67.10.4752-4759.2001

Mathew J, John N, Vazhacharickal P, and NKS (2016). Comparative study of the activity of amylase produced by *Aspergillus niger t*hough solid state fermentation (SSF*) usi*ng various starchy materials. *5*, 2319–3824.

Mehmood T, Ahmed A, Ahmad A, Ahmad MS, and Sandhu MA (2018). Optimization of mixed surfactants-based β-carotene nanoemulsions using response surface methodology: An ultrasonic homogenization approach. *Food Chemistry*, *253*, 179–184. https://doi.org/10.1016/j.foodchem.2018.01.136

Mehta D, and Satyanarayana T (2016). Bacterial and archaeal α-amylases: diversity and amelioration of the desirable characteristics for industrial applications. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01129

Meng L, Li Z, Liu L, Chen X, JunjunWu, Li W, Zhang X, and Dong M (2020). Lead removal from water by a newly isolated *Geotrichum candidum* LG-8 from Tibet kefir milk and its mechanism. *Chemosphere*, *259*, 127507. https://doi.org/10.1016/j.chemosphere.2020.127507

Menon G, Mody K, Datta S, and Jha B (2014). Characterization of alkaliphilic, surfactant stable and raw starch digesting β-amylase from *Bacillus subtilis* strain JS-16. *Journal of Microbial and Biochemical Technology*, *S8*. https://doi.org/10.4172/1948-5948.S8-002

Miranda I, Simões R, Medeiros B, Nampoothiri KM, Sukumaran RK, Rajan D, Pereira H, and Ferreira-Dias, S. (2019). Valorization of lignocellulosic residues from the olive oil industry by production of lignin, glucose and functional sugars. *Bioresource Technology*, *292*, 121936. https://doi.org/10.1016/j.biortech.2019.121936

Misbah A, Aouine M, Raouan SE, Lekbach Y, Ettadili H, Koraichi SI, and Tahri Jouti MA (2019). Microorganisms isolated from moroccan olive-mill wastes: Screening of their enzymatic activities for biotechnological use. *European Scientific Journal ESJ*, *15*(30). https://doi.org/10.19044/esj.2019.v15n30p464

Mo K, and Hayashida S (1988). Production and characteristics of two types of endocellulase from *Geotrichum candidum*. *Agricultural and Biological Chemistry*, *52*(7), 1675–1682. https://doi.org/10.1080/00021369.1988.10868913

Mojsov K, Andronikov D, Janevski A, Jordeva S, Kertakova M, Golomeova S, Gaber S, and Ignjatov I (2018). Production and application of α-amylase enzyme in textile industry. *Tekstilna Industrija*, *66*(1), Article 1.

Mostafa FA, Wehaidy HR, El-Hennawi HM, Mahmoud SA, Sharaf S, and Saleh SAA (2024). Statistical optimization of α-amylase production from novel local isolated *Bacillus* spp. NRC1 and its textile applications. *Catalysis Letters*. https://doi.org/10.1007/s10562-023-04545-2

Oussadi MI, and Kitouni M (2015). Statistical optimization of cultural conditions of an halophilic alpha-amylase production by halophilic *Streptomyces* sp. grown on orange

waste powder. *Biocatalysis and Agricultural Biotechnology*, *4*(4), 685–693. https://doi.org/10.1016/j.bcab.2015.08.011

Muhammad A, Bokhari SAI, Vernoux JP, Ali MI, Faryal R, Desmasures N, and Imran M (2019). Purification, characterization and thermodynamic assessment of an alkaline protease by *Geotrichum Candidum* of dairy origin. *Iranian Journal of Biotechnology*, *17*(2), e2042. https://doi.org/10.21859/ijb.2042

Muralikrishna G, and Nirmala M (2005). Cereal α-amylases—An overview. *Carbohydrate Polymers*, *60*(2), 163–173. https://doi.org/10.1016/j.carbpol.2004.12.002

Muriithi J, Matofari J, and Nduko J (2021). Amylolytic microorganisms from diverse tropical environments: Isolation, identification, and amylase production. *Applied Research*, *1*. https://doi.org/10.1002/appl.202100007

Nair HP, and Bhat SG (2020). Arabian sea metagenome derived—α-amylase P109 and its potential applications. *Ecological Genetics and Genomics*, *16*, 100060. https://doi.org/10.1016/j.egg.2020.100060

Najmalddin H, Yurdugül S, and Hamzah H (2023). Screening of enzyme activities for improvement of bread quality by potato peel addition to the yeast growth medium. *Food Bioscience*, *51*, 102239. https://doi.org/10.1016/j.fbio.2022.102239

Niyomukiza S, Owino W, Mathara JM, and Maina N (2023). Isolation, purification and biochemical characterization of alkaline α-amylase from *Bacillus subtilis* strain W3SFR5 isolated from kitchen Wastes. *Applied Food Biotechnology*, *10*(1), Article 1. https://doi.org/10.22037/afb.v10i1.39495

Obi CN, Okezie O, and Ezugwu AN (2019). Amylase production by solid state fermentation of agro-industrial wastes using *Bacillus* species. *European Journal of Nutrition and Food Safety*, 408–414. https://doi.org/10.9734/ejnfs/2019/v9i430087

Okpara MO (2022). Microbial enzymes and their applications in food industry: A mini-review. *Advances in Enzyme Research*, *10*(01), 23–47. https://doi.org/10.4236/aer.2022.101002

Olakusehin VO, and Oyedeji O (2022). Production of α-amylase from *Aspergillus flavus* S2-OY using solid substrate fermentation of potato (*Solanum tuberosum L.*) peel. *International Journal of Biological and Chemical Sciences*, *15*(5), 1950–1967. https://doi.org/10.4314/ijbcs.v15i5.21

Omidiji O, Amund O, Braimoh A, Ilori M (1997). Production of α-amylase in a corn steep liquor-soya bean meal medium by a strain of *Bacillus stearothermophilus*. *Microbios*, *90*, 155–162.

Owolabi OE, Olaniyi OO, and Akinyosoye FA (2023). Catalytic properties of purified alpha amylase from *Aspergillus flavus* cultivated on low-cost agricultural substrate. *Revista Facultad Nacional de Agronomía Medellín*, *76*(1), Article 1. https://doi.org/10.15446/rfnam.v76n1.100842

Oyedeji O, Olakusehin VO, and Okonji RE (2023). A thermostable extracellular α-amylase from *Aspergillus flavus* S2-OY: Purification, characterisation and application in raw starch hydrolysis. *Biocatalysis and Biotransformation*, *41*(3), 174–186. https://doi.org/10.1080/10242422.2021.2005032

Pandey A (2003). Solid-state fermentation. *Biochemical Engineering Journal*, *13*(2), 81–84. https://doi.org/10.1016/S1369-703X(02)00121-3

Paulová L, and Brányik T (2013). *Advanced Fermentation Processes* (pp. 89–105). https://doi.org/10.1201/b15426-6

Pellis A, Cantone S, Ebert C, and Gardossi L (2018). Evolving biocatalysis to meet bioeconomy challenges and opportunities. *New Biotechnology*, *40*, 154–169. https://doi.org/10.1016/j.nbt.2017.07.005

Perkins V, Vignola S, Lessard MH, Plante PL, Corbeil J, Dugat-Bony E, Frenette M, and Labrie S (2020). Phenotypic and genetic characterization of the cheese ripening yeast *Geotrichum candidum. Frontiers in Microbiology, 11*. https://doi.org/10.3389/fmicb.2020.00737

Péter G, and Rosa C (Eds.). (2006). *Biodiversity and Ecophysiology of Yeasts*. Springer-Verlag. https://doi.org/10.1007/3-540-30985-3

Phonlamai A, Kingkaew T, Prajanket P, Sakdapetsiri C, Krajangsang S, Kitpreechavanich V, and Lomthong T (2024). Raw starch degrading alkaline α-amylase from *Geobacillus kaustophilus* TSCCA02: Production, characterization, and its potential for application as a detergent additive. *Journal of Basic Microbiology*, *n/a*(n/a). https://doi.org/10.1002/jobm.202300653

Piegza M, Witkowska D, and Stempniewicz R (2014). Enzymatic and molecular characteristics of *Geotrichum candidum* strains as a starter culture for malting. *Journal of the Institute of Brewing*, *120*(4), 341–346. https://doi.org/10.1002/jib.167

Pitt JI , and Hocking AD (2009). Primary keys and miscellaneous fungi. In J. I. Pitt and A. D. Hocking (Eds.), *Fungi and Food Spoilage* (pp. 53–143). Springer US. https://doi.org/10.1007/978-0-387-92207-2_5

Plackett RL, and Burman JP (1946). The Design of optimum multifactorial experiments. *Biometrika*, *33*(4), 305–325. https://doi.org/10.2307/2332195

Prado Barragán LA, Figueroa JJB, Rodríguez Durán LV, Aguilar González CN, and Hennigs C (2016). Chapter 7—Fermentative Production Methods. In P. Poltronieri and OF. D'Urso (Eds.), *Biotransformation of Agricultural Waste and By-Products* (pp. 189–217). Elsevier. https://doi.org/10.1016/B978-0-12-803622-8.00007-0

Prajapati V, Trivedi U, and Patel K (2014). A statistical approach for the production of thermostable and alklophilic alpha-amylase from *Bacillus amyloliquefaciens* KCP2

under solid-state fermentation. *3 Biotech*, *5*. https://doi.org/10.1007/s13205-014-0213-1

Pranay K, Padmadeo SR, and Prasad B (2019). Production of amylase from *Bacillus subtilis* sp. Strain KR1 under solid state fermentation on different agrowastes. *Biocatalysis and Agricultural Biotechnology*, *21*, 101300. https://doi.org/10.1016/j.bcab.2019.101300

Premalatha A, Vijayalakshmi K, Shanmugavel M, and Rajakumar G (2022). Optimization of culture conditions for enhanced production of extracellular α-amylase using solid state and submerged fermentation from *Aspergillus tamarii* MTCC5152. *Biotechnology and Applied Biochemistry*, *70*. https://doi.org/10.1002/bab.2403

Punia Bangar S, Suri S, Trif M, and Ozogul F (2022). Organic acids production from lactic acid bacteria: A preservation approach. *Food Bioscience*, *46*, 101615. https://doi.org/10.1016/j.fbio.2022.101615

Raina D, Kumar V, and Saran S (2022). A critical review on exploitation of agro-industrial biomass as substrates for the therapeutic microbial enzymes production and implemented protein purification techniques. *Chemosphere*, *294*, 133712. https://doi.org/10.1016/j.chemosphere.2022.133712

Ramachandran S, Patel AK, Nampoothiri KM, Chandran S, Szakacs G, Soccol CR, and Pandey A (2004). Alpha amylase from a fungal culture grown on oil cakes and its properties. *Brazilian Archives of Biology and Technology*, *47*(2), 309–317. https://doi.org/10.1590/S1516-89132004000200019

Rathod BG, Pandala S, and Poosarla VG (2023). A novel halo-acid-alkali-tolerant and surfactant stable amylase secreted from halophile *Bacillus siamensis* F2 and its application in waste valorization by bioethanol production and food industry. *Applied Biochemistry and Biotechnology*, *195*(8), 4775–4795. https://doi.org/10.1007/s12010-023-04559-x

Rattray FP (2002). Cheese | Secondary Cultures. In H. Roginski (Ed.), *Encyclopedia of Dairy Sciences* (pp. 275–281). Elsevier. https://doi.org/10.1016/B0-12-227235-8/00064-X

Reddy NS, Nimmagadda A, and Rao KRSS (2003). An overview of the microbial -amylase family. *African Journal of Biotechnology*, *2*(12), Article 12. https://doi.org/10.4314/ajb.v2i12.14896

Redhead SA, and Malloch DW (1977). The Endomycetaceae: New concepts, new taxa. *Canadian Journal of Botany*, *55*(13), 1701–1711. https://doi.org/10.1139/b77-198

Redhead S, and Malloch D (2011). The Endomycetaceae: New concepts, new taxa. *Canadian Journal of Botany*, *55*, 1701–1711. https://doi.org/10.1139/b77-198

Riyadi FA, Alam Z, Salleh N, and Salleh HM (2017). Thermostable and organic solvent tolerant lipase producing fungi in solid state bioconversion of palm kernel cake. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, *25*(1), 98-105

Robyt JF, and Ackerman RJ (1971). Isolation, purification, and characterization of a maltotetraose-producing amylase from *Pseudomonas stutzeri*. *Archives of Biochemistry and Biophysics*, *145*(1), 105–114. https://doi.org/10.1016/0003-9861(71)90015-4

Rodionova NA, Dubovaia NV, Eneĭskaia EV, Martinovich LI, Gracheva IM, and Bezborodov AM (2000). Purification and characteristic of endo-(1—4)-beta-xylanase from *Geotrichum candidum* 3C. *Prikladnaia biokhimiia i mikrobiologiia*, *36*(5), 535–540.

Rodionova NA, Dubovaia NV, Odintsova TI, Gracheva IM, and Bezborodov AM (2002). Isolation of endo-1,4-beta-xylanase from *Geotrichum candidum* 3C with various ability of sorption on an insoluble substrate. *Prikladnaia Biokhimiia I Mikrobiologiia*, *38*(5), 490–494.

Rosliza R, and wan nik wan sani (2010). Improvement of corrosion resistance of AA6061 alloy by tapioca starch in seawater. *Current Applied Physics*, *10*, 221–229. https://doi.org/10.1016/j.cap.2009.05.027

Roy JK, Rai SK, and Mukherjee AK (2012). Characterization and application of a detergent-stable alkaline α-amylase from *Bacillus subtilis* strain AS-S01a. *International Journal of Biological Macromolecules*, *50*(1), 219–229. https://doi.org/10.1016/j.ijbiomac.2011.10.026

Sacristán N, González L, Castro JM, Fresno JM, and Tornadijo ME (2012). Technological characterization of *Geotrichum candidum* strains isolated from a traditional Spanish goats' milk cheese. *Food Microbiology*, *30*(1), 260–266. https://doi.org/10.1016/j.fm.2011.10.003

Saha SP, Ghosh S, Mazumdar D, Ghosh S, Ghosh D, Sarkar MM, and Roy S (2023). Valorization of banana peel into α-amylase using one factor at a time (OFAT) assisted artificial neural network (ANN) and its partial purification, characterization, and kinetics study. *Food Bioscience*, *53*, 102533. https://doi.org/10.1016/j.fbio.2023.102533

Sahni TK, Goel A, and Tech B (2015). Microbial enzymes with special reference to α-amylase. *Biology, Chemistry, Environmental Scienc*e

Sahoo RK , Sahu A, and Subudhi E (2020). Bioremediation of hydrocarbon using bacterial lipase from waste biomass. *Iranian Journal of Science and Technology, Transactions A: Science*, *44*. https://doi.org/10.1007/s40995-020-00935-

Saitou N, and Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Salah HA, Temerk HA, Salah NA, Alshehri SRZ, Al-Harbi JA, Mawad AMM, Khaled KAM, Hesham AEL, and Amein KA (2021). Production and optimization of xylanase and α-amylase from non-saccharomyces yeasts (*Pichia membranifaciens*). *Journal of Pure and Applied Microbiology*, *15*(1), 452–461. https://doi.org/10.22207/JPAM.15.1.43

Samanta A, and Jana SC (2024). Optimization of cold active amylase production by mesophilic *Bacillus cereus* RGUJS2023 under submerged fermentation. *Journal of Environmental Biology*, *45*, 16–24. https://doi.org/10.22438/jeb/45/1/MRN-5167

Sampaio JP, Gadanho M, Santos S, Duarte FL, Pais C, Fonseca A, and Fell JW (2001). Polyphasic taxonomy of the basidiomycetous yeast genus *Rhodosporidium*: *Rhodosporidium kratochvilovae* and related anamorphic species. *International Journal of Systematic and Evolutionary Microbiology*, *51*(Pt 2), 687–697. https://doi.org/10.1099/00207713-51-2-687

Santorelli M, Maurelli L, Pocsfalvi G, Fiume I, Squillaci G, La Cara F, del Monaco G, and Morana A (2016). Isolation and characterisation of a novel alpha-amylase from the extreme haloarchaeon *Haloterrigena turkmenica*. *International Journal of Biological Macromolecules*, *92*, 174–184. https://doi.org/10.1016/j.ijbiomac.2016.07.001

Santos LFS (2021). Decyl oleate production by enzymatic esterification using *Geotrichum candidum* lipase immobilized on a support prepared from rice husk. *Biocatalysis and Agricultural Biotechnology*, 36 (2):102142. https://doi-org.sndl1.arn.dz/10.1016/j.bcab.2021.102142

Sarabia LA, Ortiz MC, and Sánchez MS (2020). Response surface methodology. In S. Brown, R. Tauler, and B. Walczak (Eds.), *Comprehensive Chemometrics (Second Edition)* (pp. 287–326). Elsevier. https://doi.org/10.1016/B978-0-12-409547-2.14756-0

Saranraj P, and Stella D (2013). Fungal amylase—A review. *International Journal of Microbiological Research*, *4* (2):203-211 https://doi.org/10.5829/idosi.ijmr.2013.4.2.75170.

Saryono S, Devi S, Nugroho TT, Fadhila WF, Lorenita L, Nasution FS, and Suraya N (2023). Amylase enzyme production with variation of carbon sources and molecular identification of thermophilic fungus *Aspergillus* sp. LBKURCC304 from Bukik

Gadang, West Sumatra, Indonesia. *Biodiversitas Journal of Biological Diversity*, *24*(2), Article 2. https://smujo.id/biodiv/article/view/12185

Sethi BK, Nanda PK, Sahoo S, and Sena S (2016). Characterization of purified α-amylase produced by *Aspergillus terreus* NCFT 4269.10 using pearl millet as substrate. *Cogent Food and Agriculture*, *2*(1), 1158902. https://doi.org/10.1080/23311932.2016.1158902

Shad M, Hussain N, Usman M, Akhtar MW, and Sajjad M (2023). Exploration of computational approaches to predict the structural features and recent trends in α-amylase production for industrial applications. *Biotechnology and Bioengineering*, *120*(8), 2092–2116. https://doi.org/10.1002/bit.28504

Sharif S, Shah AH, Fariq A, Jannat S, Rasheed S, and Yasmin A (2023). Optimization of amylase production using response surface methodology from newly isolated thermophilic bacteria. *Heliyon*, *9*(1), e12901. https://doi.org/10.1016/j.heliyon.2023.e12901

Shimada Y, Sugihara A, Iizumi T, and Tominaga Y (1990). CDNA cloning and characterization of *Geotrichum candidum* lipase II. *The Journal of Biochemistry*, *107*(5), 703–707. https://doi.org/10.1093/oxfordjournals.jbchem.a123112

Shimada Y, Sugihara A, Tominaga Y, Iizurni T, and Susumu T (1989). CDNA molecular cloning of *Geotrichum candidum* lipase. *The Journal of Biochemistry*, *106*(3), 383–388. https://doi.org/10.1093/oxfordjournals.jbchem.a122862

Sidebottom CM, Charton E, Dunn PPJ, Mycock G, Davies C, Sutton JL, Macrae AR, and Slabas AR (1991). *Geotrichum candidum* produces several lipases with markedly different substrate specificities. *European Journal of Biochemistry*, *202*(2), 485–491. https://doi.org/10.1111/j.1432-1033.1991.tb16398.x

Simair AA, Qureshi AS, Khushk I, Ali CH, Lashari S, Bhutto MA, Mangrio GS, and Lu C (2017). Production and partial characterization of *α* -amylase enzyme from *Bacillus* sp.

BCC 01-50 and potential applications. *BioMed Research International*, *2017*, 1–9. https://doi.org/10.1155/2017/9173040

Singh P, Kashyap K, and Singh S (2014). Isolation, purification and characterization of oil degrading bacteria from different oil cake samples. *International Journal of Bioinformatics and Biological Science*, *2*(3and4), 189. https://doi.org/10.5958/2321-7111.2014.00016.X

Singh R, Bahuguna A, Chauhan P, Sharma V, Kaur S, Singh S, and Khan A (2016). Production, purification and characterization of thermostable α-amylase from soil isolate *Bacillus* sp. Strain B-10. *Journal of BioScience and Biotechnology*, 5(1): 37-43.

Singh R, Gupta N, Goswami VK, and Gupta R (2006). A simple activity staining protocol for lipases and esterases. *Applied Microbiology and Biotechnology*, *70*(6), 679–682. https://doi.org/10.1007/s00253-005-0138-z

Singh R, Kim S, Kumari A, and Mehta P (2022). An overview of microbial α-amylase and recent biotechnological developments. *Current Biotechnology*, *11*. https://doi.org/10.2174/2211550111666220328141044

Singh R, Langyan S, Sangwan S, Gaur P, Khan FN, Yadava P, Rohatgi B, Shrivastava M, Khandelwal A, Darjee S, and Sahu PK (2022). Optimization and production of alpha-amylase using *Bacillus subtilis* from apple peel: Comparison with alternate feedstock. *Food Bioscience*, *49*, 101978. https://doi.org/10.1016/j.fbio.2022.101978

Singhania RR, Patel AK, Soccol CR, and Pandey A (2009). Recent advances in solid-state fermentation. *Biochemical Engineering Journal*, *44*(1), 13–18. https://doi.org/10.1016/j.bej.2008.10.019

Skaltsounis AL, Argyropoulou A, Aligiannis N, and Xynos N (2015). 11—Recovery of high added value compounds from olive tree products and olive processing by products. In

D. Boskou (Ed.), *Olive and Olive Oil Bioactive Constituents* (pp. 333–356). AOCS Press. https://doi.org/10.1016/B978-1-63067-041-2.50017-3

Slater CS, Huggins TG, Brooks CA, and Hollein HC (1986). Purification of alkaline phosphatase by ultrafiltration in a stirred batch cell. *Separation Science and Technology*, *21*(6–7), 575–590. https://doi.org/10.1080/01496398608056136

Souza PMde, and Magalhães PdeOe (2010). Application of microbial α-amylase in industry—A review. *Brazilian Journal of Microbiology*, *41*, 850–861. https://doi.org/10.1590/S1517-83822010000400004

Spiess C, Happersberger HP, Glocker MO, Spiess E, Rippe K, and Ehrmann M (1997). Biochemical characterization and mass spectrometric disulfide bond mapping of periplasmic α-amylase MalS of *Escherichia coli*. *Journal of Biological Chemistry*, *272*(35), 22125–22133. https://doi.org/10.1074/jbc.272.35.22125

Splittstoesser DF, Bowers J, Kerschner L, and Wilkison M (1980). Detection and incidence of *Geotrichum candidum* in frozen blanched vegetables. *Journal of Food Science*, *45*(3), 511–513. https://doi.org/10.1111/j.1365-2621.1980.tb04089.x

Srimathi S, and Jayaraman G (2005). Effect of glycosylation on the catalytic and conformational stability of homologous α-amylases. *The Protein Journal*, *24*(2), 79–88. https://doi.org/10.1007/s10930-004-1514-8

Sudan SK, Kumar N, Kaur I, and Sahni G (2018). Production, purification and characterization of raw starch hydrolyzing thermostable acidic α-amylase from hot springs, India. *International Journal of Biological Macromolecules*, *117*, 831–839. https://doi.org/10.1016/j.ijbiomac.2018.05.231

Sundarram A, and Murthy TPK (2014). α-Amylase production and applications: A review. *Journal of Applied and Environmental Microbiology*, *2*(4), Article 4. https://doi.org/10.12691/jaem-2-4-10

Swetha S, Varma A, and Padmavathi T (2014). Statistical evaluation of the medium components for the production of high biomass, α-amylase and protease enzymes by *Piriformospora indica* using Plackett–Burman experimental design. *3 Biotech*, *4*(4), 439–445. https://doi.org/10.1007/s13205-013-0168-7

Tiwari S, Srivastava R, Singh C, Shukla K, Singh R, Singh P, Singh R, Singh N, and Sharma R (2015). Amylases: An overview with special reference to alpha amylase. *Journal of Global Biosciences*, *4*, 1886–1901.

Toksoy Oner E (2006). Optimization of ethanol production from starch by an amylolytic nuclear petite *Saccharomyces cerevisiae* strain. *Yeast (Chichester, England)*, *23*(12), 849–856. https://doi.org/10.1002/yea.1399

Turchetti B, Goretti M, Branda E, Diolaiuti G, D'Agata C, Smiraglia C, Onofri A, and Buzzini P (2013). Influence of abiotic variables on culturable yeast diversity in two distinct Alpine glaciers. *FEMS Microbiology Ecology*, *86*(2), 327–340. https://doi.org/10.1111/1574-6941.12164

Unnikrishnan B, Lien CW, Chu HW, and Huang CC (2021). A review on metal nanozyme-based sensing of heavy metal ions: Challenges and future perspectives. *Journal of Hazardous Materials*, *401*, 123397. https://doi.org/10.1016/j.jhazmat.2020.123397

Vachher M, Sen A, Kapila R, and Nigam A (2021). Microbial therapeutic enzymes: A promising area of biopharmaceuticals. *Current Research in Biotechnology*, *3*, 195–208. https://doi.org/10.1016/j.crbiot.2021.05.006

Vadkertiová R, and Sláviková E. (1995). Killer activity of yeasts isolated from the water environment. *Canadian Journal of Microbiology*, *41*(9), 759–766. https://doi.org/10.1139/m95-105

Van Bogaert INA, De Maeseneire SL, and Vandamme EJ (2009). Extracellular polysaccharides produced by yeasts and yeast-like fungi. In T. Satyanarayana and G.

Kunze (Eds.), *Yeast Biotechnology: Diversity and Applications* (pp. 651–671). Springer Netherlands. https://doi.org/10.1007/978-1-4020-8292-4_29

Vyas S, and Chhabra M (2017). Isolation, identification and characterization of *Cystobasidium oligophagum* JRC1: A cellulase and lipase producing oleaginous yeast. *Bioresource Technology*, *223*, 250–258. https://doi.org/10.1016/j.biortech.2016.10.039

Wadia T, and Jain S (2017). Isolation, screening and identification of lipase producing fungi from oil contaminated soil of Shani Mandir Ujjain. *International Journal of Current Microbiology and Applied Sciences*, *6*, 1872–1878. https://doi.org/10.20546/ijcmas.2017.607.223

Walker GM (2009). Yeasts. In M. Schaechter (Ed.), *Encyclopedia of Microbiology (Third Edition)* (pp. 478–491). Academic Press. https://doi.org/10.1016/B978-012373944-5.00335-7

Williams KF, Agwa OK, Abu GO, and Akaranta O (2021). Screening, characterization and identification of sophorolipid-producing yeast isolated from palm oil effluent polluted soil. *Journal of Advances in Microbiology*, 1–16. https://doi.org/10.9734/jamb/2021/v21i1230409

Wolf B (2015). *Geotrichum candidum*: A yeast holding on to its moldy past. *MicrobialFoods.Org*. https://microbialfoods.org/geotrichum-candidum-mold-transition/

Wouters JTM, Ayad EHE, Hugenholtz J, and Smit G (2002). Microbes from raw milk for fermented dairy products. *International Dairy Journal*, *12*(2), 91–109. https://doi.org/10.1016/S0958-6946(01)00151-0

Yaghmour MA, Bostock RM, Morgan DP, and Michailides TJ (2012). Biology and sources of inoculum of *Geotrichum candidum* causing sour rot of peach and nectarine fruit in California. *Plant Disease*, *96*(2), 204–210. https://doi.org/10.1094/PDIS-05-11-0391

Zaier H, Maktouf S, Roussos S, and Rhouma A (2021). Filamentous fungi isolated from Tunisian olive mill wastes: Use of solid-state fermentation for enzyme production. *Notulae Botanicae Horti Agrobotanica Cluj-Napoca*, *49*(1), 12125. https://doi.org/10.15835/nbha49112125

Zaky AS, Greetham D, Louis EJ, Tucker GA, and Du C (2016). A New Isolation and Evaluation Method for Marine-Derived *Yeast spp.* With Potential Applications in Industrial Biotechnology. *Journal of Microbiology and Biotechnology* , *26*(11), 1891–1907. https://doi.org/10.4014/jmb.1605.05074

Zhang F, Xinyi Y, Geng L, Zhang Z, Yin Y, and Li WJ (2015). Purification and characterization of a novel and versatile α-amylase from thermophilic *Anoxybacillus* sp. YIM 342. *Starch - Stärke*, *68*. https://doi.org/10.1002/star.201400056

Zhang X, Li C, Chen X, Chio C, Shrestha S, and Qin W (2021). *Bacillus velezensis* identification and recombinant expression, purification and characterization of its alpha-amylase. *Fermentation*, *7*, 227. https://doi.org/10.3390/fermentation7040227

Zhang XY, Zong MH, and Li N (2017). Whole-cell biocatalytic selective oxidation of 5-hydroxymethylfurfural to 5-hydroxymethyl-2-furancarboxylic acid. *Green Chemistry*, *19*(19), 4544–4551. https://doi.org/10.1039/C7GC01751K

Zhou Q, and Qiu H (2019). The mechanistic impact of n-glycosylation on stability, pharmacokinetics, and immunogenicity of therapeutic proteins. *Journal of Pharmaceutical Sciences*, *108*(4), 1366–1377. https://doi.org/10.1016/j.xphs.2018.11.029

Annexes

Annex 1: Culture media preparation

- **Yeast Peptone Glucose (YPG)**

Glucose 20 g

Peptone 20 g

Yeast extract 5 g

Distilled water 1000 ml

pH adjusted to 6.5. The medium is sterilized for 20 minutes at 12

- **Yeast Peptone Glucose Agar (YPGA)**

Glucose 20 g

Peptone 20 g

Yeast extract 5 g

Agar 20 g

Distilled water 1000 ml.

pH adjusted to 6.5. The medium is sterilized for 20 minutes at 120°C

- **Yeast Extract Peptone Starch Agar (YPSA)**

Dissolve in 1 liter of distilled water:

Soluble starch 20 g

Peptone 10 g

Yeast extract 5 g

Agar 20 g

Autoclave at 121°C for 15 min.

- **Lugol solution**

Iodine (I_2): 1 g ,

Potassium iodide (K_I): 2 g

Distilled water: 100 ml

- **Sabouraud Dextrose Agar (SDA)**

Dissolve in 1 liter of distilled water:

Casein peptone	5 g
Meat peptone	5 g
Glucose	40 g
Agar	20 g

Autoclave at 121°C for 15 min.

- **Yeast Malt Extract Agar (YMA)**

Dissolve in 1 liter of distilled water:

Yeast extract	3 g
Malt extract	3 g
Peptone	5 g
Glucose	10 g
Agar	20 g

Autoclave at 121°C for 15 min.

- **Yeast Extract Peptone CMC Agar (YPCA)**

CMC	20 g
Peptone	10 g
Yeast extract	5 g
Agar	20 g

Autoclave at 121°C for 15 min.

- **Red Congo solution**

1% in distilled water.

- **Phenol red Olive Oil Agar (POA)**

Dissolve in 1 liter of distilled water:

Olive oil	10 ml
Red phenol	0.1 g
$CaCl_2$	5 g
Agar	20 g

pH adjusted to 7.3-7.4 with NaOH (0.1N).

Autoclave at 121°C for 15 min.

- **Tween Peptone Agar (TPA)**

Dissolve in 1 liter of distilled water:

Peptone	10 g
$CaCl_2$	0.1 g
Tween 80	10 ml
Agar	20 g

Autoclave at 121°C for 15 min.

Annex 2: D1/D2 Sequence (ARNr 26S gene)

>**Geotrichum_candidum_PO27**
AAAATAATCAAAACTTTTAACAATGGATCTCTTGGTTCTCGTATCGATGAAGAAC
GCAGCGAAACGCGATATTTCTTGTGAATTGCAAAAGTGAATCATCAGTTTTTGAA
CGCACATTGCACTTTGGGGTATCCCCCAAAGTATACTTGTTTGAGCGTTGTTTCTC
TCTTGGAATTGCATTGCTTTTCTAAAATTTCGAATCAAATTCGTTTGAAAAACAAC
ACTATTCAACCTCAAATCAAGTAGGATTACCCGCTGAACTTAAGCATATCAATAA
GCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCA
AAAGCTCAAATTTGAAATCGGCCACCAGGTCGAGTTGTAATTTGTAGATGGTATC
TGGAGAGCGGATTAAAGTCTGTTGGAACACAGCGCCTTAGAGGGTGACAGCCCC
GTAAAATCTATTCTCATTGTAAGATACTTTCGAAGAGTCGAGTTGTTTGGGAATG
CAGCTCTAAGTGGGAGGTAAATTCCTTCTAAAGCTAAATATTGACGAGAGACCGA
TAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAA
AAAGTACGTGAAATTGTTAAAAGGGAAGGGTATTGAATCAGACTTGGTGCTGTTG
TTCAACTGTGTTTCGGCATAGTGTACTCAGCAGTACTAGG**.**

Annex 3: Analytic methods

1. Determination of amylase activity (Bernfeld 1955 method)

- **Principle**

α-Amylase activity is assessed by the hydrolysis of starch enzyme extract. Hydrolyzed starch release maltose, which is determined by Bernfeld's (1955) method using 3,5-dinitrosalycilic acid. (DNSA) as an amylase inhibitor. The reaction is colorimetric and the intensity of coloration is proportional to the amount of maltose released.

- **Substrate preparation**

Dissolve 1 g of potato starch in 100 ml phosphate buffer pH 5.

- **Preparation of DNSA reagent**

- Dissolve 1g of 3,5-dinitrosalycilic acid in 20 ml 2N NaOH and 50 ml distilled water
- Add 30g double tartrate of sodium and potassium, make up to 100 ml with distilled water, filter.

- **Maltose calibration curve**

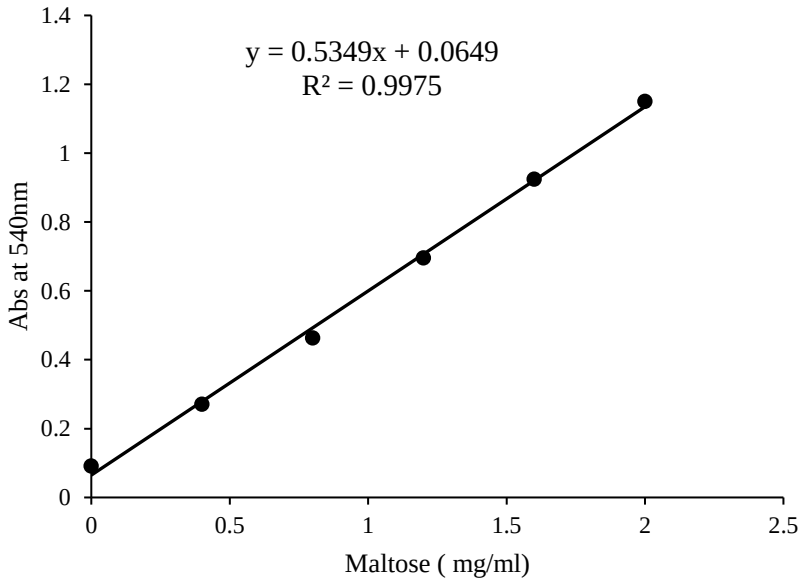

Figure 1: Maltose calibration curve .

2. Determination of carbohydrate content (Dubois 1956 method)

- **Reagents**

- Concentrated sulfuric acid d = 1.84

- 5% phenol

- **Procedure**

- To 1ml of diluted enzyme extract, add 1ml of 5% phenol and 5ml of concentrated concentrated sulfuric acid

- After stirring, let stand for 10 min.

- Incubate in water bath at 30°C for 20-30 min.

- Read at 488 nm against a control and a 100 µg/ml glucose standard.

- **Glucose calibration curve**

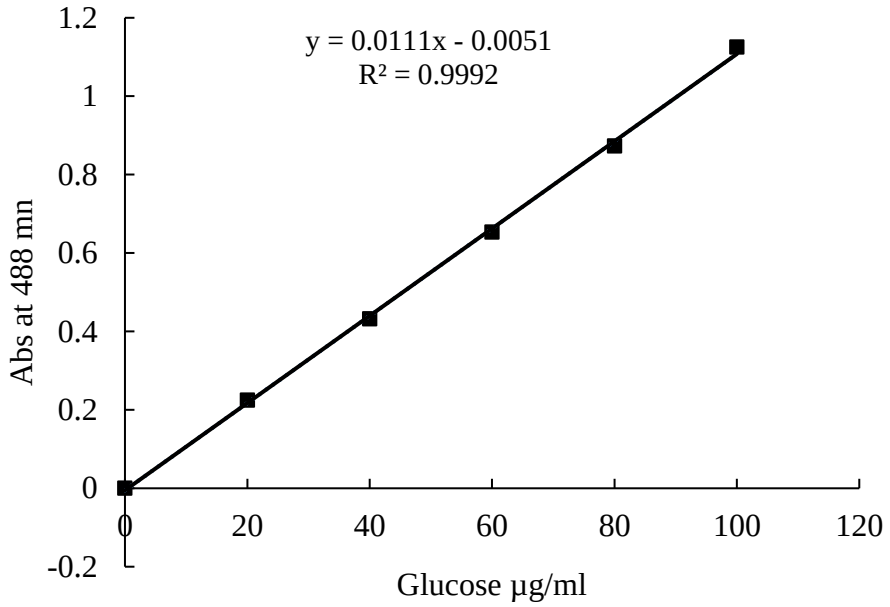

Figure 2: Glucose calibration curve.

- **Mannose calibration curve**

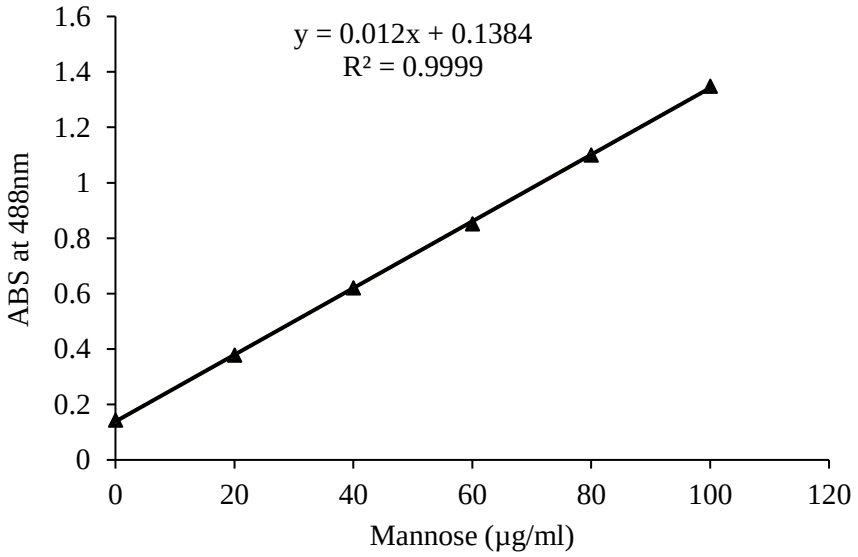

Figure 3: Mannose calibration curve.

3. Determination of protein concentration (Bradford)

- **BSA calibration curve**

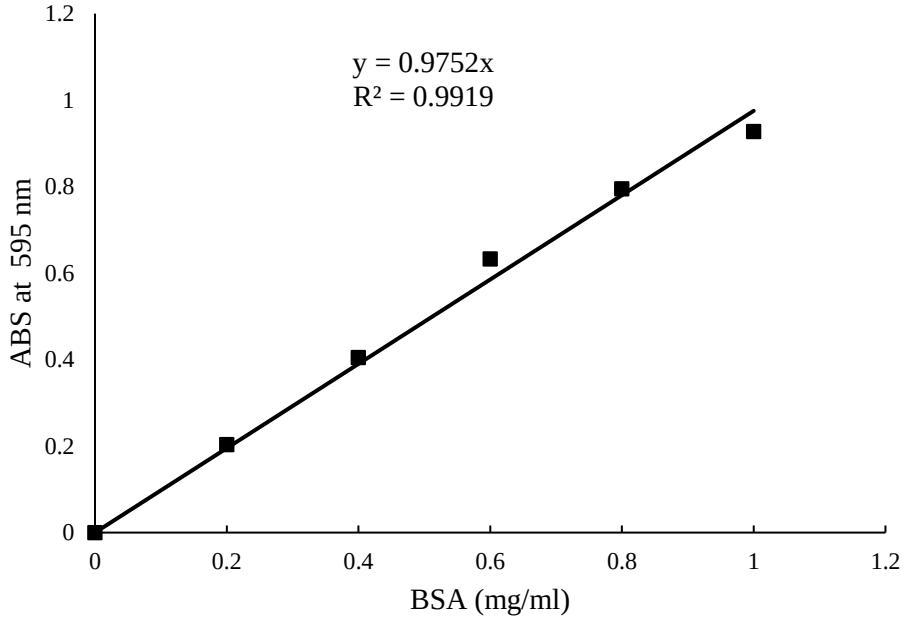

Figure 4: BSA calibration curve .

Annex 4: Electrophoresis protocol

- **Electrophoresis protocol**

1. Using a shaker, 100 microliters of purified enzyme extract was mixed with 50µl of SDS extraction solution for 20 minutes, then incubated at 65°C for 30 minutes.
2. It was then centrifuged for 1 minute at 10,000rpm. Next, 100µl of sample was loaded into wells ready for migration in 3.48% concentration gel and 14 % separation gel.
3. Revelation was initiated with a mixture of Coomassie blue and trichloroacetic acid (TCA).

Total protein extraction

1. **Preparation of stock solution**

   Mix carefully with a stirrer, Tris base buffer pH 6.8 with 20 mg of Bromophenol blue, 20 ml glycerol, distilled water, and SDS. Then ready for use.

2. **Preparation of extraction solution**

   Mix 2.125 ml of stock solution, then make up to 5 ml with distilled water.

3. **Preparation of migration buffer:**

4. Mix 28.22g of glycine, 6g of Tris, and 2g of SDS in 1L of distilled water. Then, make up to 2L.

- **SDS-polyacrylamide gel electrophoresis**

Table 1: Composition of the prepared gel.

Gel	Concentration	Separation
Acrylamide 40%	1 ml	17 ml
Bisacrylamide	0.5 ml	7.5 ml
H20	1.405 ml	4.3 ml
Tris-HCL Buffer	pH :8.8 ml	pH :6.8
Degassing the gel		
SDS 10%	0.4 ml	0.5 ml
APS 1%	0.5 ml	1.25 ml
TEMED	11.25µL	25µL

Annex 5: Statistical results (ANOVA, Tukey tests)

4. Enzyme characterizations (ANOVA test and Tukey method)

Table 2: One-way ANOVA test for temperature effect

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	7	136,545	19,5065	56,56	0,000
Error	8	2,759	0,3449		
Total	15	139,305			

Table 3 : Grouping best temperature using the Tukey method and 95% confidence

Temperature	N	Mean	Grouping			
70	2	30,706	A			
100	2	28,357		B		
80	2	26,85		B	C	
90	2	26,344		B	C	
60	2	26,176		B	C	
50	2	25,337			C	
40	2	21,646				D
30	2	21,48				D

Means that do not share a letter are significantly different.

Table 4: One-way ANOVA test for pH effect

Source	DF	Adj SS	Adj MS	F-Value	P-Value
pH	8	152,651	19,0814	23,36	0,000
Error	9	7,352	0,8169		
Total	17	160,003			

Table 5 : Grouping best pH using the Tukey method and 95% confidence

pH	N	Mean	Grouping				
5	2	21,813	A				
7	2	19,1706	A	B			
10	2	18,038		B	C		
6	2	17,367		B	C		
9	2	16,70		B	C	D	
4	2	14,514			C	D	E
11	2	13,591				D	E
8	2	13,0041					E
3	2	12,836					E

Means that do not share a letter are significantly different.

Table 6: One-way ANOVA test for metal ions effect

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Metal ions	10	3,72887	0,372887	62,92	0,000
Error	11	0,06519	0,005926		
Total	21	3,79406			

Table 7 : Grouping metal ions using the Tukey method and 95% confidence

Metal ions	N	Mean	Grouping			
Mg^{2+}	2	1,7839	A			
Hg^{2+}	2	1,1719		B		
Fe^{2+}	2	1,1719		B		
Ca^{2+}	2	1,1420		B		
K^{+}	2	1,0375		B		
Control	2	1,000		B		
Na^{+}	2	0,9703		B		
Ni^{2+}	2	0,9554		B		
Zn^{2+}	2	0,9330		B		

Cu^{2+}	2	0,4926			C	
Mn^{2+}	2	0,045				D

Means that do not share a letter are significantly different.

Table 8: One-way ANOVA test for chemical reagents effect

Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
chemical reagents	10	1,04308	0,104308	17,48	0,000
Error	11	0,06563	0,005966		
Total	21	1,10871			

Table 9 : Grouping chemical reagents using the Tukey method and 95% confidence

Chemical reagents	**N**	**Mean**	**Grouping**			
Tween 80	2	1,4406	A			
Triton X-100	2	1,1868	A	B		
DMSO	2	1,17934	A	B		
Ethanol	2	1,1793	A	B		
Acetone	2	1,1495	A	B	C	
Urea	2	1,135	A	B	C	
Control	2	1,000		B	C	
SDS	2	0,9853		B	C	
EDTA	2	0,9703		B	C	
Tween 20	2	0,8509			C	D
β-Mercaptoethanol	2	0,5524				D

Means that do not share a letter are significantly different.

Table10: Effect of different metals and chemical reagents .on purified α-amylase

Metals and Chemicals	Relative activities		
	1	2	Ecart type
Control	100,00%	100,00%	0
NaCl	95,54%	97,03%	0,021
$FeCl_3$	118,68%	117,19%	0,021
KCl	100,02%	103,75%	0,052
$CuSO_4$	46,28%	49,26%	0,042
$NiSo_4$	97,78%	95,54%	0,031
$ZnCl_2$	94,80%	93,30%	0,021
$MnSO_4$	-10,45%	4,48%	0,211
$MgSO_4$	183,62%	178,39%	0,073
$CaCl_2$	108,98%	114,20%	0,073
$HgCl_2$	113,46%	117,19%	0,052
Urea	123,91%	113,46%	0,147
EDTA	97,03%	97,03%	0
SDS	101,51%	98,53%	0,042
Tween 20	91,81%	85,09%	0,095
Tween 80	140,33%	144,06%	0,052
Triton X-100	125,40%	118,68%	0,095
B-Mercaptoethanol	59,71%	55,24%	0,063
Ethanol	126,89%	117,93%	0,126
DMSO	118,68%	117,93%	0,010
Acetone	117,19%	114,95%	0,0316

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Research article

Extracellular enzymes producing yeasts study: cost-effective production of α-amylase by a newly isolated thermophilic yeast *Geotrichum candidum* PO27

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Abstract: Enzymes are biocatalysts mainly used for their industrial potential in various applications. The present study aims to understand the enzyme production for biotechnological interest from a local yeast strain. From 100 isolates obtained from various biotopes, 78 strains were selected for their enzymatic heritage. Screening of α-amylase, lipase/esterase, and cellulase activities by rapid plate detection methods was carried out and the PO27 yeast was selected for its high capacity to produce α-amylase. In addition, this yeast strain exhibited good lipolytic and esterolytic activities, as well as low cellulase activity. A sequence analysis of the D1/D2 region of the 26S ribosomal RNA (26S rRNA) and a study of morphological characteristics identified the PO27 strain as *Geotrichum candidum*. The production of α-amylase has been studied in solid medium fermentation using various natural substrates without any supplementation such as olive pomace, potato peels, leftover bread, and mastic cake. *G. candidum* PO27 showed an improved production of α-amylase with olive pomace, thus reaching approximately 180.71 U/g. To evaluate the ability of this isolate to produce α-amylase in submerged fermentation, multiple concentrations of olive pomace substrate were tested. The best activity of submerged fermentation was statistically compared to the solid-state fermentation result in order to select the appropriate fermentation type. A high significant difference was found to rank the 6% olive pomace medium as the best substrate concentration with 34.395 U/mL of α-amylase activity. This work showed that the new isolate *Geotrichum candidum* PO27 has a better potential to produce

α-amylase at a low cost in solid-state fermentation compared to submerged fermentation. Optimization conditions for PO27 α-amylase production through solid-state fermentation were achieved using a one factor at a time (OFAT) approach. The findings revealed that a high temperature (60 ℃), an acidic pH, malt extract, and soluble starch were the highly significant medium components for enhancing α-amylase production. The use of olive pomace waste by *Geotrichum candidum* PO27 is expected to be effective in producing an industrially useful α-amylase.

Keywords: *Geotrichum candidum* PO27; α- amylase; Olive pomace; SSF; SmF; OFAT

Abbreviations: SmF: submerged fermentation; SSF: state fermentation; OP: olive pomace; OS: olive forest soil; DNSA: dinitro salicylic acid; TW: thermal water; MC: mastic oil cake; VS: vegetable smen; RW: olive rinse water; YPSA: yeast extract peptone soluble starch agar; YPDA: yeast extract peptone dextrose agar; YM: yeast extract malt extract; SDA: sabouraud dextrose agar; YGA: yeast extract glucose agar; YPCA: yeast extract peptone carboxymethyl cellulose agar; TPA: tween peptone agar; AI: amylolytic index; OFAT: one factor at a time; NCBI: National Center for Biotechnology Information.

1. Introduction

The demand for industrial enzymes is continuously growing worldwide, in which the net worth of approximately $0.31 billion in 1960 was raised to $4.9 billion in 2015, to $6 billion in 2020, and is expected to reach more than $9 billion USD by 2027. This has led to significant success for many enzyme companies [1–3]. These enzymes are widely secreted by plants, animals, and microorganisms. However, microbial enzymes have received more attention due to their easy availability, rapid growth, and an active and stable nature, as well as their high yield on inexpensive media and in a shorter time and a straightforward production by recombinant DNA technology using microbes as the host cells [4,5]. Most microorganisms are unable to produce enzymes under challenging conditions that induce microorganism toxicity. However, some microorganisms with thermostable enzyme systems have undergone various adaptations that allow them to grow and produce enzymes under harsh conditions, mainly temperature and pH, to be commercially attractive [6,7].

Recently, several studies have been initiated to isolate many potential enzyme-producing microorganisms, including bacteria, fungi, and yeast strains from several sources. They can be isolated from soil, water [8,9], hot springs [10], fruits, wastes from the palm oil plant [11], marine environments [12], and oil mill effluent [13,14]. In recent years, yeasts have emerged as a major source of enzymes, in addition to their high lipid production capacity, short fermentation cycles, independence of climatic growth, low pH value, and growth capabilities on a large variety of substrates [15]. It has been found that yeasts producing enzymes such as α-amylase, lipase, cellulase, and esterase are found everywhere in nature. These enzymes are applied in several industrial processes, such as textiles, leather, paper and pulp, research and development, pharmaceutical, agriculture, detergent, waste, biorefineries, photography, and food industries [3]; with these attractive remarkable characteristics, they are particularly considered as an ideal biocatalyst.

The production of enzymes such as α-amylase using synthetic media is very expensive and an uneconomical, low-cost medium is required to meet the demand of industries. For these reasons, researchers have investigated agro wastes such as potato peels, bread waste, and oil cakes [16–18] as

an alternative substrate to replace the high-cost media employed through submerged fermentation (SmF) and solid-state fermentation (SSF). In addition, it helped to reduce the environmental pollution caused by their disposal in the fields, which could create a serious environmental problem due to the phytotoxic nature of their compounds. To reduce the cost of enzymes, it would be interesting to isolate local enzyme-producing yeast strains with new characteristics. The selected *Geotrichum candidum* PO27 can use various wastes as an alternative carbon source for α-amylase production under SSF and SmF, which is an economic advantage since the application of these enzymes is increasing in several industries. However, a few investigations on α-amylase production from *G. candidum* have been performed, more specifically, using agro-industrial waste. Therefore, to fill these scientific gaps, this work aims to isolate yeasts with significant enzymatic potential for biotechnological use, to produce inexpensive α-amylase using locally available and environmentally harmful substrates, and to optimize fermentation conditions by studying their effects on the enzymatic production of the strain and improving the yield and quality of the product.

2. Materials and methods

2.1. Reagents

All bacteriological grade growth and selective media were prepared in the GMA laboratory. Dehydrated ingredients for prepared media and soluble potato starch were purchased from Panreac Química SA (Spain). Soluble starch was purchased from Biochem (France), and carboxymethyl cellulose and 3,5-Dinitrosalicylic acid were procured from Sigma-Aldrich (Germany). Other reagents and organic salts in this work were of analytical grade and commercially available (Biochem Chemopharma Co**)**. Olive oil was obtained from a local olive oil mill (Mila, Algeria).

2.2. Sampling

An olive forest soil (OS) sample was collected in the region of Skikda, Algeria after removing approx. 5 cm of soil from the surface. A 95 ℃ thermal water (TW) sample was taken from a hot spring called Hammam El Dabbagh in the region of Guelma. Olive pomace Sigoise variety (OP), its rinse water (RW), and a mastic oil cake (MC) (*Pistacia lentiscus L*) were collected from an oil mill in the region of Mila and Skikda, respectively; these wastes were used as a source of yeast as well as a substrate for α-amylase production. Vegetable smen (VS) is one of the traditional Algerian butter products, and was purchased from supermarkets in the region of Constantine, Algeria. All samples were collected with a sterile spatula into appropriately pre-sterilized bottle containers and stored in a cool place (4 ℃) until use. The pH of the samples was determined using a pH meter (GLP 21, Spain) at the laboratory level. Each sample was designated alphabetically.

2.3. Isolation and purification

Two methods were tested for the isolation of yeast strains. The method of successive dilutions on yeast peptone dextrose Agar (YPDA) medium [19] included spreading with a glass spreader, alongside an incubation period between three to seven days at 28 ℃ (Memmert INB 400, Germany). After macroscopic and microscopic observation, purification was carried out by streaking on yeast extract

malt extract agar (YMA) medium and incubated until pure isolates were obtained. The pure strains were stored at 4 ℃ in sabouraud dextrose agar (SDA) supplemented with chloramphenicol and in yeast extract malt extract (YM) medium supplemented with 30% glycerol at −20 ℃.

The enrichment method was also used for isolation. This is a procedure in which 2 mL of thermal water inoculum as the source of yeast strains was incubated in 50 mL YM broth using 250 mL Erlenmeyer flasks at 30 ℃ on a rotary shaker (Stuart SI500, UK) at 150 rpm for 24 hours. After incubation, the spread was performed on YM agar, YGA, and SDA, and incubated at 28 ℃ for three to seven days [10].

2.4. Screening of amylolytic strains

Starch-degrading yeasts were screened by growing in yeast extract peptone soluble starch agar (YPSA) medium containing 1% of starch as the sole carbon source [20] using the colony picking method with four quadrant isolates. The clearance zone was detected after flooding the plates with an iodine solution (1% I_2, 2% KI), which reacts with the color of the undegraded starch.

2.5. Screening of cellulolytic strains

Cellulase producing yeasts were screened by growing in the yeast extract peptone carboxymethyl cellulose agar (YPCA) medium containing 1% carboxymethylcellulose (CMC) (Sigma) using the streak method with four quadrant isolates [21]. One % of Congo red solution and NaCl (1 M) for 20 minutes were used to reveal their ability to degrade cellulose. Cellulolytic activity was determined by observing a pale-yellow color around the yeast colonies.

2.6. Esterase activity on Tween-80 agar medium

All strains were standardized (4 McFarland) in sterile distilled water, and a total of 62 µL of the yeast suspension on 6 mm wells was inoculated into Tween-80 agar medium [21,22]. The inoculated plates were incubated at 28 ℃ for 72 hours. A clear zone in the form of crystals on the Tween peptone agar (TPA) medium was observed around the colony, thus indicating either esterase or lipase production.

2.7. Lipase activity on olive oil agar medium

All strains were incubated on an olive oil agar medium using red phenol as a pH indicator [23,24]. The lipolytic activity was observed by the appearance of a yellow zone. The zones of clearing, which measured <20 mm, 20–30 mm, and >30 mm, were noted as weak (+), moderate (++), and high (+++) activities, respectively.

2.8. Enzymatic index

The enzymatic activity index is a practical tool to improve the selection and comparison of the enzyme production of different microbial isolates. It is calculated as the relationship between the halo size and the degradation capacity of the microorganisms using the following formula:

$$Enzymatic\ index\ (EI) = \frac{Turbid\ zone\ diameter\ (mm)}{colony\ diameter\ (mm)} \quad [25]$$

2.9. Characterization and identification of the selected yeast strain

Yeasts with a high capacity in α-amylase production and diverse enzyme activities were identified. The identification of the selected strain was based on morphological characteristics and molecular identification. A macroscopic observation of the colonies was performed on YPD agar [26] with the naked eye, following several characteristics such as color, texture, appearance, and growth time. A microscopic observation (Leica DM1000, Germany) was performed with the 40 and 100x objectives based on the shape and mode of reproduction.

The selected yeast was subjected to molecular identification based on the amplification of the D1/D2 region of the 26S ribosomal RNA (26S rRNA) gene. DNA extraction was performed by the method of Sampaio et al. [27]. DNA amplification was performed by the PCR method using the forward primer V9G (5′-TGCGTTGATTACGTCCCTGC-3′) and the reverse primer RLR3R (5′ -GGTCCGTGTTTCAAGAC-3). Sequencing of the 600–650 bp region was performed using the forward primer NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and the reverse primer NL4 (5′-GGTCCGTGTTTCAAGACGG-3′; Sigma-Aldrich Co) as mentioned in the study of Turchetti et al. [28]. The PCR products were sequenced using a commercial sequencing facility (Macrogen, Amsterdam, Netherlands). The NCBI GenBank public database was used to search for sequences compatible with the obtained sequences. Then, a phylogenetic tree was constructed by the Neighbor-joining method with 1000 bootstraps [29]. The phylogenetic distances were calculated with the Kimura-2-parameter [30]. The evolutionary analyses were carried out in Molecular Evolutionary Genetics Analysis 11 (MEGA 11) (MEGA Software, Pennsylvania, USA).

2.10. Production of α-amylase by the selected strain

2.10.1. Substrate

The fermentation studies were performed using various wastes as a basal substrate. In our study, various locally collected agricultural wastes and industrial effluents were collected from oil mills. Residues such as potato peels, leftover bread, olive pomace, and mastic oil cake were not readily available in dried form. Therefore, these collected substrates were air dried to remove the moisture content; then, grinding was performed using an electric grinder and sieved to provide a particle size ≤1 mm prior to use.

2.10.2 Inoculum

For inoculum preparation, YPDA medium was used to inoculate the selected strain *Geotrichum candidum* PO27; sterile distilled water was added after incubation for 48 h at 30 ℃, and the cell suspension was obtained after removing the colony from the agar using a sterile Pasteur pipette. Direct counting was performed using a Thoma cell counter. An inoculum of 10^7 cells/mL was used [31].

2.10.3 Solid state fermentation (SSF) and extraction

Solid substrates were weighed (10 g) and the desired moisture level was maintained (60%, v/w) and inoculated with 10^7 cells/mL for an incubation period of 72 h at 30 ℃. An enzyme extraction was performed by adding a 0.1 M phosphate buffer, pH 7.0, with a ratio of 1:5 (w/v). The solution was vortexed for 5 min; crude enzyme was collected from the supernatant after centrifugation (Sigma 3K15, Germany) at 10,000 rpm for 10 min at 4 ℃ [32]. The best substrate medium was selected for submerged fermentation.

2.10.4 Submerged fermentation (SmF)

Medium of olive pomace substrate (2%–8% in distilled water) was distributed at a rate of 40 mL per 250 mL Erlen Meyer flask. After sterilization at 120 ℃ for 20 min and cooling, the media were inoculated (10^7 cells/mL) and incubated at 30 ℃ for 72 h at 150 rpm. After fermentation, the mixture was centrifuged at 10,000 rpm, 4 ℃, for 10 min and the supernatant was used to determined α-amylase activity.

2.10.5 Optimization of Cultivation Conditions for α-amylase production by one factor at a time (OFAT) approach

The one factor at a time (OFAT) method was used to estimate the effects of the following conditions on a-amylase production by the *Geotrichum candidum* PO27 yeast strain: temperature, pH, nitrogen, and carbon sources. Different fermentation temperatures, from 30 ℃ to 65 ℃, were studied to obtain the optimal incubation temperature for α-amylase. The effect of pH was studied at pH values of 4.0, 5.0, and 6.0. Buffer systems were used at a concentration of 0.1 M sodium citrate buffer to pH 4.0 and 5.0, and a sodium potassium phosphate buffer to pH 6. To determine the effects of different carbon and nitrogen sources, the basic medium was supplemented with 1% of different organic and inorganic nitrogen sources, such peptone, malt extract, yeast extract, meat extract, corn steep, KNO_3, $NaNO_3$, NH_4Cl, and $(NH_4)_2SO_4$. Additionally, glucose, galactose, sucrose, fructose, maltose, lactose, soluble starch, and potato starch were added at 1% to the basal fermentation media. Cultures were incubated for a period of 72 hours.

2.10.6 Alpha amylase assay

The 3,5-Dinitrosalicylic acid (DNSA) method was used to quantify the produced α-amylase enzyme [33,34]. Approximately 0.5 mL of the extract was incubated for 30 min at 40 ℃ with 0.5 mL of substrate (1%) prepared in (0.1 M) phosphate buffer, pH 5. The reaction was stopped by DNSA, followed by heating at 100 ℃ for 10 min. After cooling in an ice bath, 10 mL of distilled water was added. The absorbance was determined at 540 nm (VWR UV-1600PC Spectrophotometer, China). One unit of amylase activity is defined as the amount of amylase, which releases one μmole of maltose per min under the assay conditions. The enzymatic activity was expressed as U/gds (gram of dry substrate). All the cultures were duplicates and the results are the mean.

2.10.7 Statistical analysis

The results were processed by the Minitab 19 software (Minitab, LLC, Pennsylvania, USA), which is a software designed for data analysis. All experiments were performed in duplicates, data were analyzed using an one-way ANOVA, followed by Turkey's multiple comparison test, and the statistical significance level of 0.05 was chosen. All graphics were created using GraphPad Prism 10 (Graphpad Software, LLC, Boston, USA).

3. Results and discussion

3.1. Isolation

In this study, a total of six samples were collected from several regions to obtain yeasts capable of producing α-amylase, cellulase, esterase, and lipolytic enzymes. After isolation, the pH results were taken based on the pH measurement of the samples' stock solution using a pH meter.

It was found that the four samples, namely olive pomace, rinse water, mastic oil cake, and vegetable smen, had acidic pH values of 4.40, 6.30, 6.20, and 6.33, respectively. The pH of the thermal water was neutral at 6.98, while the pH value of the olive forest soil was basic at 7.39. Generally, yeasts prefer a slightly acidic environment, and their optimal pH is between 4.5 and 5.5. Nevertheless, they grow between a wide range of pH values and adapt to pH levels between 3–10. In addition, several species can grow at strongly acidic pH values as low as 1.5 [35]. Generally, habitats with basic pH values are quite difficult for yeast life, while some species can grow successfully at pH levels of 10 or above [36].

From 100 isolated yeast strains, 78 yeast strains were selected for their rapid growth on YPDA (Figure 1). Based on a higher incidence, 27 yeast strains were selected from the olive pomace of isolated yeasts and six yeasts strains were isolated from olive rinse water (RW). Misbah et al's research [37] proved that RW, OP, and olive mill wastewater (OM) were favorable media for the development of enzymes producing microorganisms. Therefore, 16 strains were isolated from the soil (OS) with a similarity in colony morphology in most isolates, which agrees with the results of Williams et al. [38] who isolated 14 yeast strains obtained from palm oil impacted soil.

Twelve strains were selected from mastic oil cake (MC). To our knowledge, this source, which is used for the isolation of α-amylase, cellulase, and lipase producing yeasts, has not been previously discussed, despite their richness, as previous studies have demonstrated the importance of oily cakes as a source for the isolation of microorganisms for biotechnological use [39].

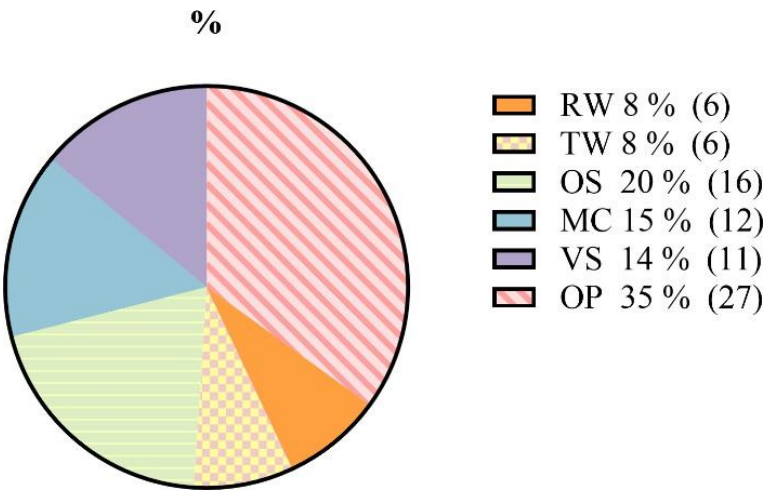

Figure 1. Frequency of isolated yeast strains.

Springs hot water is one of the major sources of thermostable enzymes produced by yeasts. The isolation of yeast from TW was unsuccessful using the successive dilution technique; alternatively, an enrichment method made it possible to obtain six heat-resistant strains. Khadka et al. [10] succeeded in isolating approximately 44 bacterial strains from Kharpani hot spring water using the enrichment method, including *Geobacillus* sp. KP43, which gave a high cellulase production. Additionally, *Geobacillus* (K1C) bacteria was isolated from Manikaran hot springs and selected for its high thermostable α-amylase production [40]. Eleven strains of VS origin were obtained, and this traditional butter has been the subject of many scientific studies. However, no detailed study has ever characterized its microbial diversity, including yeasts. In the studies cited above, the microbial diversity mainly focused on the diversity of proteolytic lactic acid bacteria and, in a few cases, yeasts in traditional Algerian butter and on yeast diversity in different types of cheeses [41,42].

3.2. Screening of enzymes producing yeasts

Hydrolysis starch by α-amylase produced a light purple area around the colony after revelation with the Lugol solution. The absence of a clear zone indicated a reaction between iodine reagents and non-hydrolyzed starches in the Starch Agar medium [43]. The iodine-starch reaction is due to the formation of helical amylose and iodine as I_3 that fills the helical nucleus [44]. Halo zone formation and amylolytic index (AI) were used as a semi-quantitative method to classify isolates as being highly amylase producers (AI > 1.5) [45]. Twenty-seven yeast strains were able to hydrolyze starch; among these strains, RW1-3, RW1-2, and PO 27, showed high amylolytic activity (Figure 2).

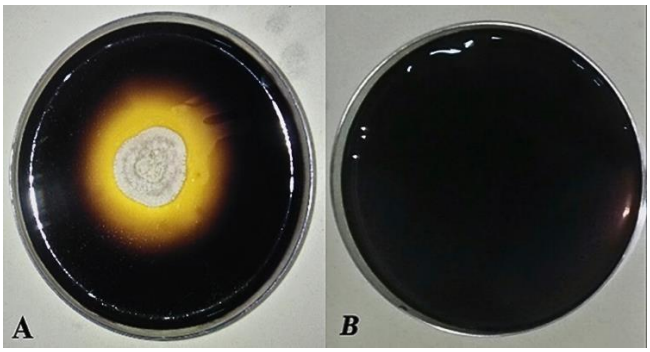

Figure 2. Amylase qualitative assay for the selected isolate PO27 (A: Presence of selected yeast, B: Absence of selected yeast).

Olive oil mixed with agar medium represents a good choice for selecting lipase-positive strains [46] with an indicator such as red phenol [47]. After incubation on an olive oil medium, the dishes displayed yellow areas around the wells due to free fatty acids released by the lipolytic organisms, which lowered the pH of the medium from phenol red to yellow, thus indicating the presence of lipase [46]. Twenty-five strains demonstrated lipolytic activity. However, the PO27 strain was the most efficient because it showed the highest diameter on olive oil (Figure 3). All other strains exhibited low and moderate lipolytic activity. The clear zone around the colony showed that the isolates were also capable of promoting cellulase hydrolysis [48]. Among the 78 yeast strains, most had no cellulase activity except five strains, namelyPO27, PO6, PO8, PO20, and RW2-2, which all showed weak activities (Figure 4). Additionally, Tween-80 was used as a substrate for esterase or lipase screening. No color is required for visualization; they provide the opaque zones around the colonies, which indicate the precipitation zones of the calcium salt, while hydrolysis indicates either esterase or lipase activity [49] (Figure 5). Seventeen strains showed esterase activity, and all species exhibited moderate and high esterase activity, including the amylolytic, cellulolytic, and lipolytic yeast isolate PO27 (Figure 6).

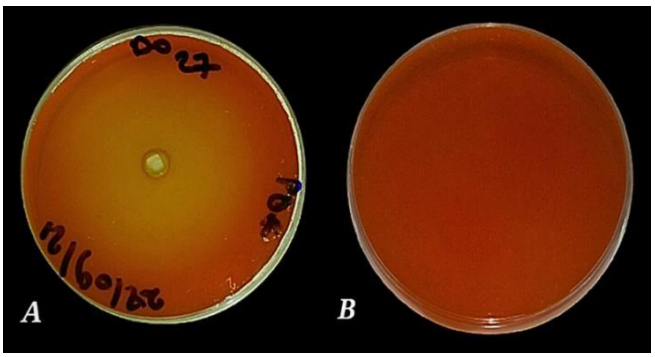

Figure 3. Lipase qualitative assay for the selected isolate PO27 (A: Presence of olive oil, B: Absence of olive oil).

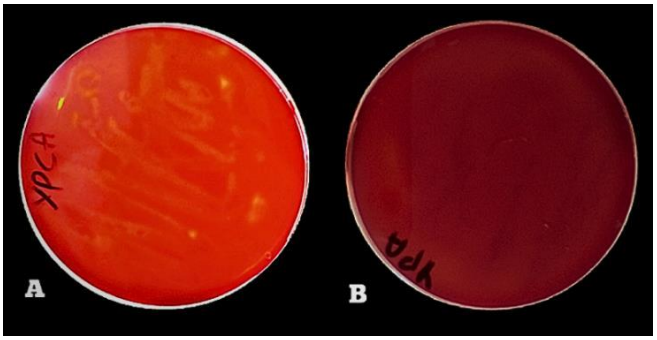

Figure 4. Cellulase qualitative assay for the selected isolate PO27 (A: Presence of carboxymethyl cellulose, B: Absence of carboxymethyl cellulose).

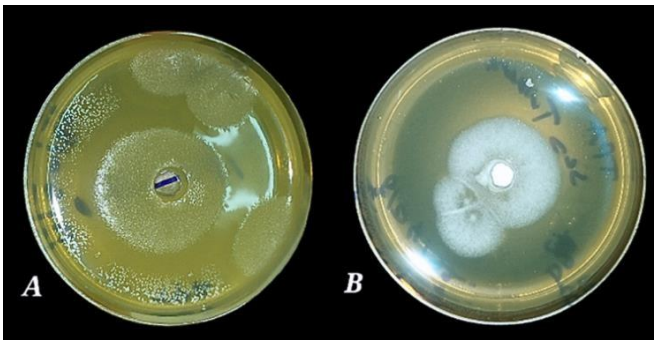

Figure 5. Esterase qualitative assay for the selected isolate PO27 (A: Presence of Tween 80, B: Absence of Tween 80).

In addition, yeast biodiversity varied between the six isolation samples (Figure 6). The results of the experiment revealed that most of them present a good biotechnological interest due to their capacity to tolerate high yeast concentrations. However, the maximum number of yeast colonies obtained from the olive pomace sample indicates that the majority of them have a more diversified and important enzymatic potential.

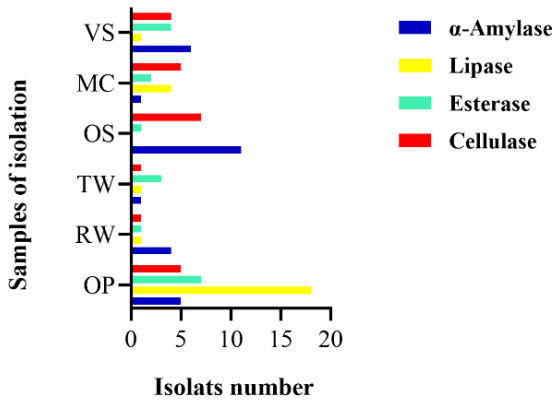

Figure 6. Distribution of enzymatic activities on the six samples. Bars: amylolytic isolated strains (blue bar), lipolytic isolated strains (yellow bar), esterolytic isolated strains (green bar), cellulolytic isolated strains (red bar).

As a result, the olive pomace origin of the yeast PO27 strain was selected for its high capacity to produce α-amylase and its enzymatic diversity profile. The PO27 isolate showed a 40 mm α-amylase halo and a 1.6 of AI, as well as a 38 mm lipase halo and 22 mm opaque halo in the Tween substrate with 1.1 of enzymatic index. Moreover, it showed a positive result with the cellulase qualitative test, and which is not interesting for industrial use because of its low activity. Following the screening and identification of the isolate, it was found that our results are consistent with those of previous studies [50], in which isolated *Geotrichum candidum* showed an ability to hydrolyze and assimilate several carbon sources including starch, Tween, casein, and CMC through their production of enzymes such as amylase, lipase (or esterase), protease, and cellulase. Furthermore, there are many reports in the literature about the production of lipases from *Geotrichum candidum* [51]. Different strains have shown the enzymatic potential of this yeast species, including *Geotrichum candidum* CMSS06, which produced α-amylase [52], *Geotrichum candidum* Strain Gad1, which is able to produce cellulase [53], *Geotrichum candidum* 3C, which produced endo-1,4-xylanase [54], *Geotrichum candidum* AA15, which also exhibited the capacity to produce pectinase [55], and *Geotrichum candidum* QAUGC01, which allowed for the production of serine alkaline protease [56].

3.3. Identification and characterization of the selected yeast strain

The species presents a yeast with a fuzzy, filamentous appearance, with a mold-like aspect, in addition to its rapid growth; the colonies are white and have a velvety white cottony mycelium, as described by Attanayaka et al. [52]. Under the light microscope, its shape is rectangular and then rounded, and showed many arthrospores, which are consistent with a previous study [57] that clearly showed conidia (arthrospores) with variable width. Based on morphological characterization, this yeast species was identified as *Geotrichum* sp. (Figure 7). The species has been confirmed by molecular identification.

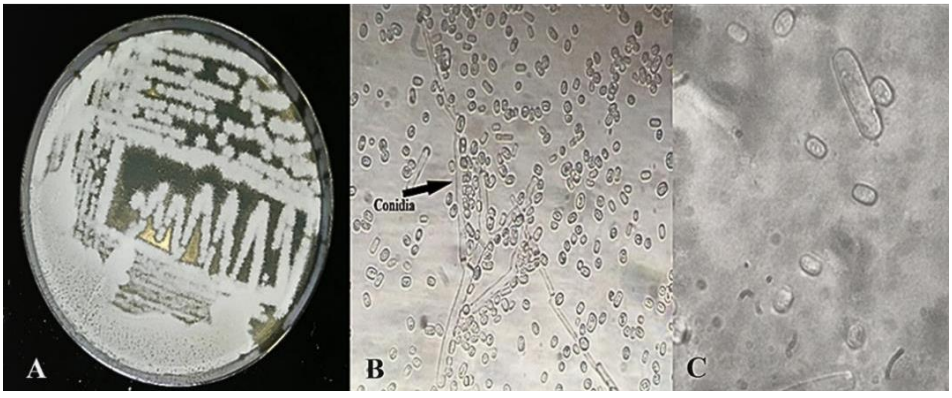

Figure 7. Morphology observation of PO27 strain cultured on YPD agar plate for 48 h at 30 °C (A: Macroscopic observation, B: Microscopic observation G:40X, C: Microscopic observation G:100X).

After molecular identification from sequencing of the D1/D2 domain of the 26S gene sequence, the length of the D1/D2 region was found to be 699 bp (is included in supplementary D1/D2 region). Sequence comparison of the PO27 isolate (GenBank accession no. PP024529) with those included in the GenBank database showed a 99.57% similarity with the *Geotrichum candidum* strain

CV2 (GenBank Accession no. KX364934) and illustrated a high similarity to its teleomorph *Galactomyces candidum.* According to the phylogenetic analysis (Figure 8), the isolate PO27 clustered in a branch near to *G. candidum* with more than 77% probability, which is well supported. Based on its morphological and molecular characteristics, we identified the isolate PO27 as *Geotrichum candidum* and not as its teleomorph *Galactomyces candidum*, since no ascospores were observed.

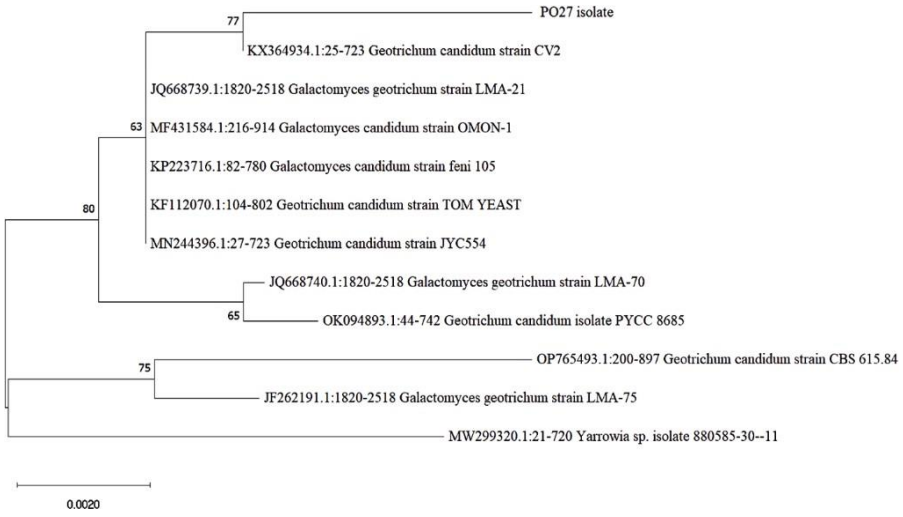

Figure 8. Phylogenetic relationships of PO27 and other closely related *Geotrichum* sp. using NJ method with bootstrap value of 1000 replicates. *Yarrowia* sp. was used as outgroup. Bootstrap values (>50%) were shown at the nodes. Scale bar, 0.002 nucleotide substitution rate units.

3.4. Production of α-amylase studies

In SSF, the most important factor is the choice of a suitable medium for the production of an enzyme. In this study, the selected strain *G. candidum* PO27 was inoculated into solid residues such as potato peels, bread leftovers, olive pomace, and mastic oil cake. Then, the produced α-amylase was quantified via the DNSAmethod. Among the four agro-substrates tested, a significant difference ($p = 0.038$) was obtained with olive pomace 180.71 U/g to be the best solid substrate for the α-amylase production, followed by potato peels 150.63 U/g, mastic oil cake 132.66 U/g, and bread leftovers 109.04 U/g (Figure 9). This can be explained by the differences in the texture and chemical composition of the substrates.

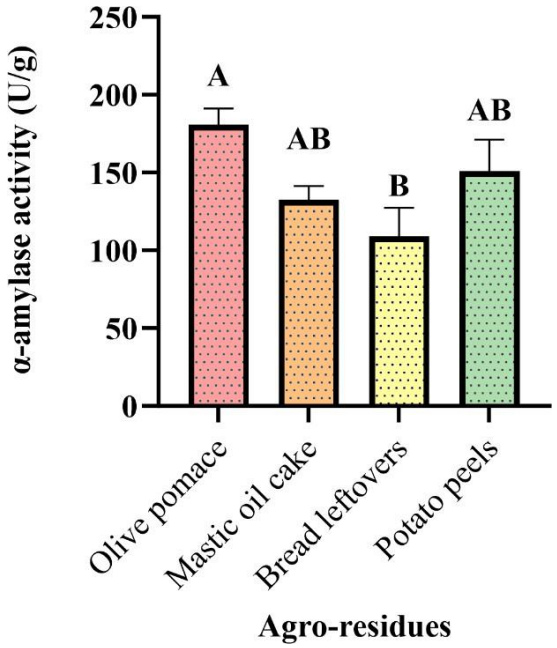

Figure 9. α-amylase production by *G. candidum* PO27 under solid-state fermentation (Tukey method: Means that do not share a letter are significantly different).

The amylases production from agro-industrial waste is intended to resolve pollution problems and to obtain a low-cost medium. Literature reports suggest that *Aspergillus awamori* isolated from olive cake can produce the highest amylase activity of 230 U/g [58]. In contrast to Obi et al. [59], the potato peel was suggested as the best substrate with the highest amylase production (2.36 U/mL) from *Bacillus subtilis*. Using various oil cakes as low-cost substrates, such as groundnut oil cake (GOC), coconut oil cake (COC), and sesame oil cake (SOC) *by Aspergillus oryzae*, Balakrishnan et al. [60] also studied α-amylase production under SSF. Their results showed that oil cake (GOC) was the best substrate for a maximal α-amylase production of 9868.12 U/g, followed by 4031.12 U/g using COC and 3068.15 U/g using SOC.

In another study, using *Bacillus subtilis* [61] to produce α-amylase, it was shown that stale bread had the highest amylolytic activity (107.3 U/min), whereas the potato peel yielded 55.5 U/min. Furthermore, Benabda et al. [62] demonstrated that SSF from *Rhizopus oryzae* produced α-amylase (100 U/g) using bread waste as a substrate, which is very similar to our SSF results on leftover bread. Agricultural substrates such as wheat bran, rice bran, maize bran, corn bran, and wheat straw have attracted attention for amylase production [63]. Moreover, the capacity of several fruit peels, including banana, orange, and pineapple peels, to provide alternative carbon sources for α-amylase production was examined [64,65]. Additionally, Singh et al. [66] found that SSF of an apple peel could yield 17468 U/L of α-amylase from the *Bacillus subtilis* BS1934 strain. In another study, watermelon rinds (WMR) were used to produce α-amylase from *Trichoderma virens* [67].

In order to choose the best conditions for enzymatic production, submerged fermentation was performed at different olive pomace substrate concentrations from 2% to 8%. The analysis of the experimental results by the ANOVA reveals that the difference in activity as a function of substrate concentration is highly significant ($p = 0.000$), where a 6% concentration was the best with 34.395

U/mL, compared to other concentrations, followed by 4% with 32.8 U/mL. In comparison, 2% and 8% exhibited low activities with 30.115 U/mL and 24.490 U/mL, respectively (Figure 10).

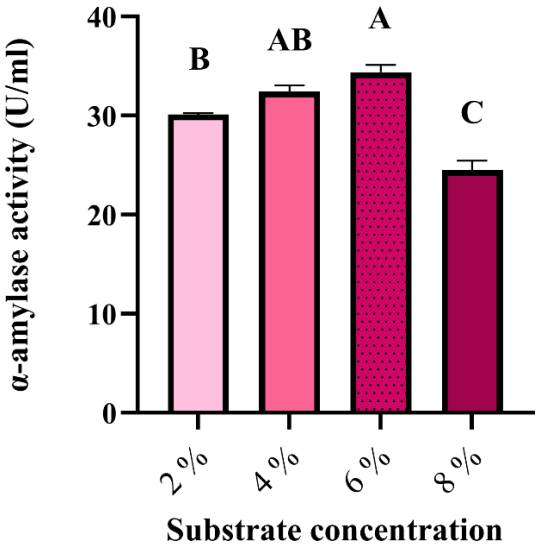

Figure 10. α-amylase production by *G. candidum* PO27 under submerged fermentation (Tukey method: Means that do not share a letter are significantly different).

Comparing our results is challenging because this is the first study to use *G. candidum* as a source of α-amylase by submerged fermentation using olive pomace. In addition, it is necessary to consider that our study is very attractive because of the dispensation of any mineral supplementation that makes it a suitable low-cost environment for α-amylase production by the *G. candidum* PO27 strain (34.395 U/mL). Using a synthetic media, the highest production of the species *Geotrichum candidum* CMSS06 reached 6.4 U/mL of α-amylase at 72 h, whereas *Aspergillus* spp. showed a maximum activity of 1.2335 U/mL at 96 h [52]. Compared to Divya and Padma [68], which used a synthetic YEPD broth supplemented with starch, the amylase activity was 130 U/mL by the isolate *Geotrichum* sp. In contrast to Falih [69], soil yeast *Geotrichum candidum* was found to have an amylase activity of 50 µg/mL using the Czapek-Dox medium, while the maximum amylolytic activity of *Geotrichum capitatum* was 34 µg/mL. Moreover, the highest growth rate of *Saccharomyces cerevisiae* has been observed in the treatment with 2% potato peels in SmF, which increased amylase activity [70]. Because of their low cost, availability, and simplicity, oil cakes such as cocos nut oil cake were used as a carbon source in liquid fermentation to produce α-amylase by *Aspergillus flavus*, which showed a higher enzyme activity (170.3 µg/mL) [71].

In recent years, the technique of the SSF process has been developed and used more extensively because of its simplicity, low cost, the simple need for fermentation equipment, improved productivity, and decreased water production [18]. As shown in Figure 11, the α-amylase activity in SSF (45.47 U/mL) was highly significant ($p = 0.038$) when compared with the activity in SmF (34.395 U/mL). This result corroborates that of Jesubunmi and Ogbonna [72], who reported that the production of glucoamylase and cellulase by both *Fusarium* sp. and *Rhizopus* sp. was significantly higher in solid state culture ($p < 0.05$) than in suspended culture. However, that did not prevent about 90% of industrially important enzymes to have traditionally been produced by SmF because of ease of handling and sterilization and a better

control of environmental factors such as temperature and pH [34]. Furthermore, Premalatha et al. [73] reported that the production of extracellular α-amylase from *Aspergillus tamarii* using wheat bran (WB) performed better in the SSF method than with SmF and achieved a higher α-amylase activity (519.40 U/g).

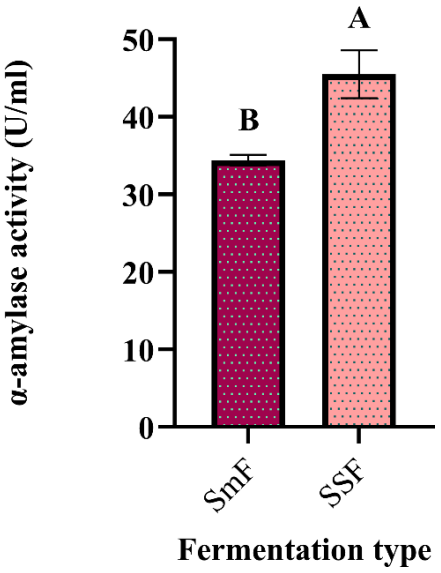

Figure 11. Comparison of α-amylase production by *G. candidum* PO27 in solid state and submerged fermentation (Tukey method: Means that do not share a letter are significantly different).

3.5. Optimization of Cultivation Conditions for α-amylase production by one factor at a time (OFAT) approach

3.5.1. Temperature effect on α-amylase production

The amylolytic strain seems to be thermophilic. The maximal activity was obtained (191.10 U/g) at 60 ℃ ($p = 0.000$), followed by 186.35 U/g at 55 ℃ (Figure 12a). Enzyme production dropped at a temperature of 65 ℃ and gave an activity level of 121.68 U/g, thus indicating the inhibition of amylase production, probably by suppressing cell viability and enzyme inactivation [74]. These results agree with several studies. Luang-In et al. [75] showed that the optimal temperature for amylase of *Bacillus* sp. 3.5AL2 was 60 ℃; moreover, Finore et al. [76] obtained maximal α-amylase secretion from *Anoxybacillusamylolyticus* at 60 ℃. The results showed that *G. candidum* PO27 α-amylase is a thermophilic enzyme with potential use in industrial processes. Microorganisms able to grow optimally at temperatures between 50 ℃ and 60 ℃ are known as moderate thermophiles. It can be assumed that moderate thermophiles, which are closely related phylogenetically to mesophilic organisms, can adapt to life in warm environments [77].

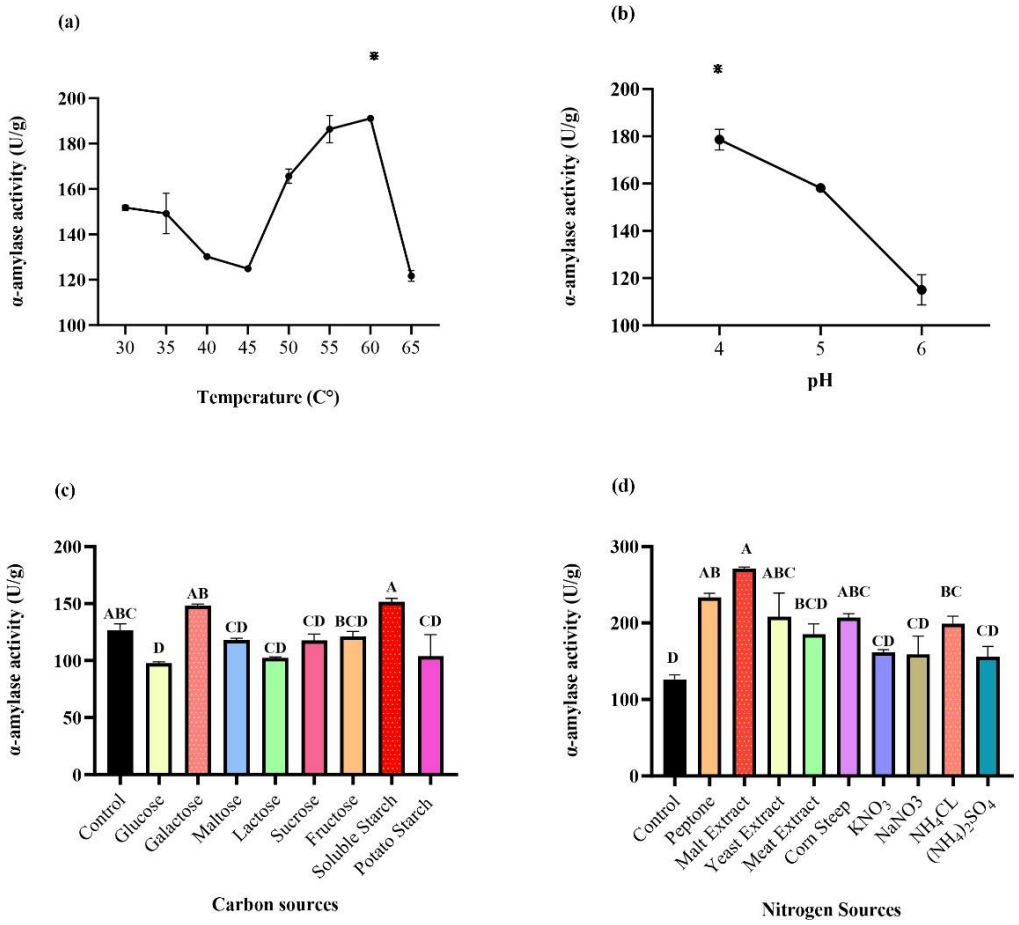

Figure 12. Optimization of α-amylase production by *Geotrichum candidum* PO27 by OFAT approach. Effect of (a) Temperature, (b) pH, (c) Carbon sources, (d) Nitrogen sources on α-amylase production, (*) indicate significant difference, (Tukey method: Means that do not share a letter are significantly different $p < 0.05$).

3.5.2. pH effect on α-amylase production

An optimal pH is an essential factor for the stability of the enzyme produced. Enzymes are pH sensitive, and therefore production process pH must be carefully controlled [78]. The extracellular α-amylase activity was significantly higher ($p = 0.002$) at a pH 4 (178,61 U/g), followed by a pH of 5 (158,145 U/g); however, the enzymatic activity was considerably lower at a pH of 6 (115,075 U/g), which indicates the acidophilic nature of the isolated *G. candidum* PO27 (Figure 12b). These results are similar to Tatsinkou Fossi et al. [79], who found that a pH 4.5 was optimal for amylase production at 30 ℃ by an isolated yeast. Additionally, Olakusehin and Oyedeji [80] revealed that the optimal pH for α-amylase production by *Aspergillus flavus* S2-OY was found to be at a pH of 5. On top of that, the pH measurement of the optimized medium of the *G. candidum* CMSS06 strain proved that the strain appropriates the acidic medium as the pH of the culture supernatant dropped from 4.0 to 3.46 [52].

3.5.3. Carbon source effect on α-amylase production

The carbon source is one of the major factors affecting enzyme production, particularly when it performs an inducer role. The α-amylase production by the G. *candidum* PO27 strain was significantly ($p = 0.000$) higher in the presence of soluble starch (151, 54 U/g), followed by galactose (148,315 U/g); however, the enzymatic activity was considerably lower in addition to the other carbon source as compared to the SSF without carbon supplementation (Figure 12c). This can be explained by the high carbohydrate content of olive pomace [81], which is effective for the α- amylase production. This enzyme is extracellular, and its production is induced by its substrate at a certain limit concentration [65]. In addition to its role as an inducer, starch stabilizes the enzyme [82]. This result is similar to Almanaa et al. [64], who produced maximal amounts of amylase from *Bacillus subtilus* D19 using starch as the carbon source. Moreover, the α-amylase production from *Streptomyces* sp. Al-Dhabi-46 was found to be maximal in the culture medium containing 1% starch as carbon source (208 ± 11.4 U/mL) [83]. In addition, galactose had a positive impact as the highest enzymatic performance (944 U/gds) by *Penicillium chrysogenum* in SSF [84].

3.5.4. Nitrogen source effect on α-amylase production

Due to the low nitrogen content of olive pomace, the α-amylase production was enhanced by the addition of various nitrogenous compounds. From the result of the Figure 12, it appears that *G. candidum* PO27 can use malt extract to significantly ($p = 0.000$) enhance α-amylase production and achieve maximal production (270,52 U/g). With few investigating the use of malt extract as a nitrogen source to improve amylase production, maximal α-amylase production using malt extract by *Pseudomonas balearica* VITPS19 has been reported by Kizhakedathil and Subathra Devi [85]. Although it served as the inorganic source, NH_4Cl gave the best α- amylase production (199.18 U/g) compared with the other inorganic sources used. Ahmed et al. [86] revealed its insignificant effect on bacterial α-amylase production.

4. Conclusions

Several yeast strains were isolated from olive pomace, olive forest soil, thermal water, olive rinse water, mastic oil cake, and vegetable smen. They were all capable of producing α-amylase, esterase, lipase, and cellulase. Among these isolates, the PO27 strain showed higher amylolytic activity and presented an important enzymatic diversity. This isolate was identified as *G. candidum* according to morphological characteristics and was confirmed by 26S rRNA gene sequencing. The present study clearly indicates that *G. candidum* PO27 isolated from olive pomace showed a significant capacity in the production of α-amylase by degrading various agro-industrial wastes as natural substrates. Among the cheap sources tested, olive pomace was best for α- amylase production, and the best activity of α-amylase was obtained by SSF compared with SmF. At the same time, the effects of physicochemical factors on PO27 α-amylase production were studied. The α-amylase production process by *G. candidum* PO27 using this waste can be optimized using a statistical design, purified, tested, and scaled up for industrial production, thus bringing environmental benefits to energy security.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

Author contributions

IC: methodology, experiment performing, data analysis, drafting the manuscript; SDD: conceptualization, methodology, supervised the project, editorial review; LB: formal analysis, review of the final version of the manuscript; TN: formal analysis, project administration. All authors approved the final version of the manuscript.

References

1. Pellis A, Cantone S, Ebert C, et al. (2018) Evolving biocatalysis to meet bioeconomy challenges and opportunities. *New Biotechnol* 40: 154–169. https://doi.org/10.1016/j.nbt.2017.07.005
2. Costa-Silva TA, Carvalho AKF, Souza CRF, et al. (2021) Enhancement lipase activity via immobilization onto chitosan beads used as seed particles during fluidized bed drying: Application in butyl butyrate production. *Appl Catal Gen* 622: 118217. https://doi.org/10.1016/j.apcata.2021.118217
3. Okpara MO (2022) Microbial enzymes and their applications in food industry: A mini-review. *Adv Enzyme Res* 10: 23–47. https://doi.org/10.4236/aer.2022.101002
4. Vachher M, Sen A, Kapila R, et al. (2021) Microbial therapeutic enzymes: A promising area of biopharmaceuticals. *Curr Res Biotechnol* 3: 195–208. https://doi.org/10.1016/j.crbiot.2021.05.006
5. Raina D, Kumar V, Saran S (2022) A critical review on exploitation of agro-industrial biomass as substrates for the therapeutic microbial enzymes production and implemented protein purification techniques. *Chemosphere* 294: 133712. https://doi.org/10.1016/j.chemosphere.2022.133712
6. Nigam P (2013) microbial enzymes with special characteristics for biotechnological applications. *Biomolecules* 3: 597–611. https://doi.org/10.3390/biom3030597
7. Dahiya D, Nigam PS (2021) An overview of three biocatalysts of pharmaceutical importance synthesized by microbial cultures. *AIMS Microbiol* 7: 124–137. https://doi.org/10.3934/microbiol.2021009

8. Trabelsi S, Ben Mabrouk S, Kriaa M, et al. (2019) The optimized production, purification, characterization, and application in the bread making industry of three acid-stable alpha- amylases isoforms from a new isolated *Bacillus subtilis* strain US586. *J Food Biochem* 43: e12826. https://doi.org/10.1111/jfbc.12826
9. Ilesanmi OI, Adekunle AE, Omolaiye JA, et al. (2020) Isolation, optimization and molecular characterization of lipase producing bacteria from contaminated soil. *Sci Afr* 8: 00279. https://doi.org/10.1016/j.sciaf.2020.e00279
10. Khadka S, Khadka D, Poudel RC et al. (2022) production optimization and biochemical characterization of cellulase from *Geobacillus sp*. KP43 Isolated from hot spring water of Nepal. *BioMed Res Int* 2022: 1–12. https://doi.org/10.1155/2022/6840409
11. Kuncharoen N, Techo S, Savarajara A, et al. (2020) Identification and lipolytic activity of yeasts isolated from foods and wastes. *Mycology* 11: 279–286. https://doi.org/10.1080/21501203.2020.1745922
12. Zaky AS, Greetham D, Louis EJ, et al. (2016) A new isolation and evaluation method for marine-derived yeast spp. with potential applications in industrial biotechnology. *J Microbiol Biotechnol* 26: 1891–1907. https://doi.org/10.4014/jmb.1605.05074
13. Kanmani P, Kumaresan K, Aravind J (2015) Utilization of coconut oil mill waste as a substrate for optimized lipase production, oil biodegradation and enzyme purification studies in *Staphylococcus pasteuri. Electron J Biotechnol* 18: 20–28. https://doi.org/10.1016/j.ejbt.2014.11.003
14. Salgado V, Fonseca C, Lopes da Silva T, et al. (2020) Isolation and identification of *Magnusiomyces capitatu*s as a lipase-producing yeast from olive mill wastewater. *Waste Biomass Valorization* 11: 3207–3221. https://doi.org/10.1007/s12649-019-00725-7
15. Vyas S, Chhabra M (2017) Isolation, identification and characterization of *Cystobasidium oligophagum* JRC1: A cellulase and lipase producing oleaginous yeast. *Bioresour Technol* 223: 250–258. https://doi.org/10.1016/j.biortech.2016.10.039
16. Ramachandran S, Patel AK, Nampoothiri KM, et al. (2004) Alpha amylase from a fungal culture grown on oil cakes and its properties. *Braz Arch Biol Technol* 47: 309–317. https://doi.org/10.1590/S1516-89132004000200019
17. Benabda O, M'hir S,Kasmi M, et al. (2019) Optimization of protease and amylase production by *Rhizopus oryzae* cultivated on bread waste using solid-state fermentation. *J Chem* 2019 : 3738181. https://doi.org/10.1155/2019/3738181
18. Olakusehin VO, Oyedeji O (2022) Production of α-amylase from *Aspergillus flavus* S2-OY using solid substrate fermentation of potato (*Solanum tuberosum L.*) peel. *Int J Biol Chem Sci* 15: 1950–1967. https://doi.org/10.4314/ijbcs.v15i5.21
19. Salah HA, Temerk HA, Salah NA, et al. (2021) Production and optimization of xylanase and α-amylase from non-saccharomyces yeasts (*Pichia membranifaciens*). *J Pure Appl Microbiol* 15: 452–461. https://doi.org/10.22207/JPAM.15.1.43
20. Lee YJ, Choi YR, Lee SY, et al. (2011) Screening wild yeast strains for alcohol fermentation from various fruits. *Mycobiology* 39: 33–39. https://doi.org/10.4489/MYCO.2011.39.1.033
21. Buzzini P, Martini A (2002) Extracellular enzymatic activity profiles in yeast and yeast-like strains isolated from tropical environments. *J Appl Microbiol* 93: 1020–1025. https://doi.org/10.1046/j.1365-2672.2002.01783.x

22. Riyadi FA, Alam Z, Salleh N, et al. (2017) Thermostable and organic solvent tolerant lipase producing fungi in solid state bioconversion of palm kernel cake. *Asia Pac J Mol Biol Biotechnol* 25: 98–105.
23. Lanka S, B TT (2018) Screening and isolation of lipase producing fungi from marine water obtained from Machilipatnam costal region. *Int J Pharmacogn Phytochem Res* 9: 7. https://doi.org/10.25258/phyto.v9i07.11157
24. Singh R, Gupta N, Goswami VK, et al. (2006) A simple activity staining protocol for lipases and esterases. *Appl Microbiol Biotechnol* 70: 679–682. https://doi.org/10.1007/s00253-005-0138-z
25. Carrim AJI, Barbosa EC, Vieira JDG (2006) Enzymatic activity of endophytic bacterial isolates of *Jacaranda decurrens* Cham. (Carobinha-do-campo). *Braz Arch Biol Technol* 49: 353–359. https://doi.org/10.1590/S1516-89132006000400001
26. Sacristán N, González L, Castro JM, et al. (2012) Technological characterization of *Geotrichum candidum* strains isolated from a traditional Spanish goats' milk cheese. *Food Microbiol* 30: 260–266. https://doi.org/10.1016/j.fm.2011.10.003
27. Sampaio JP, Gadanho M, Santos S, et al. (2001) Polyphasic taxonomy of the basidiomycetous yeast genus rhodosporidium: *Rhodosporidium kratochvilovae* and related anamorphic species. *Int J Syst Evol Microbiol* 51: 687–697. https://doi.org/10.1099/00207713-51-2-687
28. Turchetti B, Goretti M, Branda E, et al. (2013) Influence of abiotic variables on culturable yeast diversity in two distinct Alpine glaciers. *FEMS Microbiol Ecol* 86: 27–340. https://doi.org/10.1111/1574-6941.12164
29. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454
30. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16: 111–120. https://doi.org/10.1007/BF01731581
31. Dakhmouche Djekrif S, Bennamoun L, Labbani FZK, et al. (2021) An alkalothermophilic amylopullulanase from the yeast *Clavispora lusitaniae* ABS7: Purification, characterization and potential application in laundry detergent. *Catalysts* 11: 1438. https://doi.org/10.3390/catal11121438
32. Sahoo RK, Sahu A, Subudhi E (2020) Bioremediation of hydrocarbon using bacterial lipase from waste biomass. *Iran J Sci Technol Trans Sci* 44 :1287–1293. https://doi.org/10.1007/s40995-020-00935-y
33. Bernfeld P (1955) Amylases, α and β. *Meth Enzymol* 1: 149–158. https://doi.org/10.1016/0076-6879(55)01021-5
34. Oussadi MI, Kitouni M (2015) Statistical optimization of cultural conditions of an halophilic alpha-amylase production by halophilic *Streptomyces sp*. grown on orange waste powder. *Biocatal Agric Biotechnol* 4: 685–693. https://doi.org/10.1016/j.bcab.2015.08.011
35. Rosa C, and Gábor P (2006) Biodiversity and ecophysiology of yeasts. in: *The yeast handbook*, 1 Eds., Berlin; London: Springer, 155–174. https://doi.org/10.1007/3-540-30985-3
36. Buzzini P, Turchetti B, Yurkov A (2018) Extremophilic yeasts: the toughest yeasts around?. *Yeast* 35: 487–497. https://doi.org/10.1002/yea.3314

37. Misbah A, Aouine M, Raouan SE, et al. (2019) Microorganisms isolated from Moroccan olive-mill wastes: screening of their enzymatic activities for biotechnological use. *Eur Sci J ESJ* 15: 464–494. https://doi.org/10.19044/esj.2019.v15n30p464
38. Williams KF, Agwa OK, Abu GO, et al. (2021) Screening, characterization and identification of sophorolipid-producing yeast isolated from palm oil effluent polluted soil. *J Adv Microbiol* 21: 1–16. https://doi.org/10.9734/jamb/2021/v21i1230409
39. Singh P, Kashyap K, and Singh S (2014) Isolation, purification and characterization of oil degrading bacteria from different oil cake samples. *Int J Bioinforma Biol Sci* 2: 189. https://doi.org/10.5958/2321-7111.2014.00016.X
40. Sudan SK, Kumar N, Kaur I, et al. (2018) Production, purification and characterization of raw starch hydrolyzing thermostable acidic α-amylase from hot springs, India. *Int J Biol Macromol* 117: 831–839. https://doi.org/10.1016/j.ijbiomac.2018.05.231
41. Karam NE, Idoui T (2008) Lactic acid bacteria from Jijel's traditional butter: Isolation, Identification and major technological traits. *Grasas y Aceites* 59: 361–367. https://doi.org/10.3989/gya.2008.v59.i4.530
42. Bintsis T (2021) Yeasts in different types of cheese. *AIMS Microbiol* 7: 447–470. https://doi.org/10.3934/microbiol.2021027
43. Kwon YM, Choi HS, Lim JY, et al. (2020) Characterization of amylolytic activity by a marine-derived yeast *Sporidiobolus pararoseus* PH-Gra1. *Mycobiology* 48: 95–203. https://doi.org/10.1080/12298093.2020.1763100
44. Gunam IBW, Sujana IGA, Wijaya IMM, et al. (2021) Isolation and selection of amylase-producing microbes isolated from ragi tape and cassava tape available on the markets. *IOP Conf Ser Earth Environ Sci* 913: 012041. https://doi.org/10.1088/1755-1315/913/1/012041
45. Muriithi J, Matofari J, Nduko J (2021) Amylolytic microorganisms from diverse tropical environments: Isolation, identification, and amylase production. *Appl Res* 1: 1–9. https://doi.org/10.1002/appl.202100007
46. Lanka S, Latha JNL (2015) A Short review on various screening methods to isolate potential lipase producers: lipases-the present and future enzymes of biotech industry. *Int J Biol Chem* 9: 207–219. https://doi.org/10.3923/ijbc.2015.207.219
47. Bharathi D, Rajalakshmi G (2019) Microbial lipases: An overview of screening, production and purification. *Biocatal Agric Biotechnol* 22: 101368. https://doi.org/10.1016/j.bcab.2019.101368
48. Arman Z, Sondana GA, Fikriyyah NN, et al. (2020) Screening of amylolytic and cellulolytic yeast from *Dendrobium spathilingue* in Bali Botanical Garden, Indonesia. *AIP Conf Proc* 2242: 050013-1–050013-5. https://doi.org/10.1063/5.0007802
49. Wadia T, Jain SK (2017) Isolation, screening and identification of lipase producing fungi from oil contaminated soil of Shani Mandir Ujjain. *Int J Curr Microbiol Appl Sci* 6: 872–1878. https://doi.org/10.20546/ijcmas.2017.607.223
50. Zaier H, Maktouf S, Roussos S, et al. (2021) Filamentous fungi isolated from Tunisian olive mill wastes: use of solid-state fermentation for enzyme production. *Not Bot Horti Agrobot Cluj-Napoca* 49: 12125. https://doi.org/10.15835/nbha49112125
51. Hlavsová K, Zarevúcka M, Wimmer Z, et al. (2009) *Geotrichum candidum* 4013: Extracellular lipase versus cell-bound lipase from the single strain. *J Mol Catal B Enzym* 61: 188–193. https://doi.org/10.1016/j.molcatb.2009.06.012

52. Attanayaka DPSTG, De Silva SNT, Nirosha SF, et al. (2009) Isolation of raw starch hydrolysing fungi and purification of α-amylase from *Geotrichum candidum* CMSS06.*J Natl Sci Found Sri Lanka* 37: 93. https://doi.org/10.4038/jnsfsr.v37i2.1064
53. Gad AM, Suleiman WB, El-Sheikh HH, et al. (2022) Characterization of cellulase from *Geotrichum candidum* Strain Gad1 approaching bioethanol production. *Arab J Sci Eng* 47: 6837–6850. https://doi.org/10.1007/s13369-021-06391-z
54. Rodionova NA, Dubovaia NV, Eneĭskaia EV, et al. (2000) Purification and characteristic of endo-(1--4)-beta-xylanase from *Geotrichum candidum* 3C. *Prikl Biokhim Mikrobiol* 36: 535–540
55. Ahmed A, Ejaz U, Sohail M (2020) Pectinase production from immobilized and free cells of *Geotrichum candidum* AA15 in galacturonic acid and sugars containing medium. *J. King Saud Univ* 32: 952–954. https://doi.org/10.1016/j.jksus.2019.07.003
56. Muhammad A, Bokhari SAI, Vernoux JP, et al. (2019) Purification, characterization and thermodynamic assessment of an alkaline protease by *Geotrichum candidum* of dairy origin. *Iran J Biotechnol* 17: e2042. https://doi.org/10.21859/ijb.2042
57. Loo JL, Lai OM, Long K, et al. (2006) Identification and characterisation of a locally isolated lipolytic microfungus - *Geotrichum candidum*. *Malays J Microbiol* 2: 22–23. https://doi.org/10.21161/mjm.210604
58. Karam EA, Abdel Wahab WA, Saleh SAA, et al. (2017) Production, immobilization and thermodynamic studies of free and immobilized *Aspergillus awamori* amylase. *Int J Biol Macromol* 102: 694–703. https://doi.org/10.1016/j.ijbiomac.2017.04.033
59. Obi CN, Okezie O, Ezugwu AN (2019) Amylase Production by solid state fermentation of agro-industrial wastes using Bacillus species. *Eur J Nutr Food Saf* 9*:* 408–414. https://doi.org/10.9734/ejnfs/2019/v9i430087
60. Balakrishnan M, Jeevarathinam G, Kumar SKS, et al. (2021) Optimization and scale-up of α-amylase production by *Aspergillus oryzae* using solid-state fermentation of edible oil cakes. *BMC Biotechnol* 21: 33. https://doi.org/10.1186/s12896-021-00686-7
61. Khelil O, Choubane S, Maredj K, et al. (2022) UV mutagenesis for the overproduction of thermoalkali-stable α-amylase from *Bacillus subtilis* TLO3 by fermentation of stale bread: Potential application as detergent additive. *Biocatal Agric Biotechnol* 43: 02403. https://doi.org/10.1016/j.bcab.2022.102403
62. Benabda O, M'hir S, Kasmi M, et al. (2019) Optimization of protease and amylase production by *Rhizopus oryzae* cultivated on bread waste using solid-state fermentation. *J Chem* 2019: 1–9. https://doi.org/10.1155/2019/3738181
63. Pranay K, Padmadeo SR, Prasad B (2019) Production of amylase from *Bacillus subtilis sp*. strain KR1 under solid-state fermentation on different agrowastes. *Biocatal Agric Biotechnol* 21: 101300. https://doi.org/10.1016/j.bcab.2019.101300
64. Almanaa TN, Vijayaraghavan P, Alharbi NS, et al. (2020) solid-state fermentation of amylase production from *Bacillus subtilis* D19 using agro-residues. *J King Saud Univ - Sci* 32: 1555–1561. https://doi.org/10.1016/j.jksus.2019.12.011
65. Djekrif-Dakhmouche S, Gheribi-Aoulmi Z, Meraihi Z, et all. (2006) Application of a statistical design to the optimization of culture medium for α-amylase production by *Aspergillus niger* ATCC 16404 grown on orange waste powder. *J Food Eng* 73: 190–197. https://doi.org/10.1016/j.jfoodeng.2005.01.021

66. Singh R, Langyan S, Sangwan S, et al. (2022) Optimization and production of alpha-amylase using *Bacillus subtilis* from apple peel: Comparison with alternate feedstock. *Food Biosci* 49: 101978. https://doi.org/10.1016/j.fbio.2022.101978
67. Abdel-Mageed HM, Barakat AZ, Bassuiny RI, et al. (2022) Biotechnology approach using watermelon rind for optimization of α-amylase enzyme production from *Trichoderma virens* using response surface methodology under solid-state fermentation. *Folia Microbiol (Praha)* 67: 53–264. https://doi.org/10.1007/s12223-021-00929-2
68. Divya K, Padma PN (2014) Yeast isolates from diverse sources for cold- active polygalacturonase and amylase production. *Int J Sci Technol Res* 3: 145–148
69. Falih AM (1998) Effect of heavy metals on amylolytic activity of the soil yeasts *Geotrichum capitatum* and *Geotrichum candidum*. *Bioresour Technol* 66: 213–217. https://doi.org/10.1016/S0960-8524(98)00063-7
70. Najmalddin H, Yurdugül S, Hamzah H (2023) Screening of enzyme activities for improvement of bread quality by potato peel addition to the yeast growth medium. *Food Biosci* 51: 102239. https://doi.org/10.1016/j.fbio.2022.102239
71. Arunsasi M, Kani SM, Jegadeesh G, et al. (2010) Submerged fermentation of amylase enzyme by *Aspergillus flavus* using *Cocos nucifera* meal. *Kathmandu Univ J Sci Eng Technol* 6: 75–87. https://doi.org/10.3126/kuset.v6i2.4015
72. Jesubunmi C, Ogbonna J (2022) Production of cellulase and amylase enzymes in both solid and liquid states by two species of fungi. *Asian J Biotechnol Bioresour Technol* 8: 1–11. https://doi.org/10.9734/ajb2t/2022/v8i130115
73. Premalatha A, Vijayalakshmi K, Shanmugavel M, et al. (2022) Optimization of culture conditions for enhanced production of extracellular α - amylase using solid - state and submerged fermentation from *Aspergillus tamarii* MTCC5152. *Biotechnol Appl Biochem* 70: 835–845. https://doi.org/10.1002/bab.2403
74. Ahmed SA, Abdella MAA, El-Sherbiny GM, et al. (2019) Application of one–factor- at-a-time and statistical designs to enhance α-amylase production by a newly isolate *Bacillus subtilis* strain-MK1. *Biocatal Agric Biotechnol* 22: 101397. https://doi.org/10.1016/j.bcab.2019.101397
75. Luang-In V, Yotchaisarn M, Saengha W, et al. (2019) Isolation and identification of amylase-producing bacteria from soil in Nasinuan community forest, Maha Sarakham, Thailand. *Biomed Pharmacol J* 12: 1061–1068. https://doi.org/10.13005/bpj/1735
76. Finore I, Paola Di Donato IF, Poli A, et al. (2014) Use of agro waste biomass for α-amylase production by *anoxybacillus amylolyticus*: purification and properties. *J Microb Biochem Technol* 06: 320–326. https://doi.org/10.4172/1948-5948.1000162
77. Djekrif-Dakhmouche S (2016) Production and characterization of amylopullulanase from the yeast *Clavispora lusitaniae* abs7 isolated from wheat, grown and stored in arid zones, doctoral thesis. Compiegne University of Technology, (UTS), Compiegne France. 211.
78. Mathew J, John N, Vazhacharickal P, et al. (2016) Comparative study of the activity of amylase produced by *Aspergillus niger* though solid state fermentation (SSF) using various starchy materials. *Indian J Plant Sci* 5: 2319–3824
79. Tatsinkou Fossi B, Tavea F, Ndjouenkeu R (2005) Production and partial characterization of a thermostable amylase from ascomycetes yeast strain isolated from starchy soils. *Afr J Biotechnol* 4: 1684–5315.

80. Olakusehin VO, Oyedeji O (2022) Production of α-amylase from *Aspergillus flavus* S2-OY using solid substrate fermentation of potato (*Solanum tuberosum L.*) peel. *Int J Biol Chem Sci* 15: 1950– 1967. https://doi.org/10.4314/ijbcs. v15i5.21
81. Skaltsounis Al, Argyropoulou A, Aligiannis N, et al. (2015) Recovery of high added value compounds from olive tree products and olive processing by. In: Boskou, D., *Olive and Olive Oil Bioactive Constituents*, Ed., AOCS Press, 333–356. https://doi.org/10.1016/B978-1-63067-041- 2.50017-3
82. R. De Mot and H. Verachtert (1986) Secretion of alpha-amylase and multiple forms of glucoamylase by the yeast *Trichosporon pullulans*. *Can J Microbiol* 32 : 47–51 https://doi.org/ 10.1139/m86-009.
83. Al-Dhabi NA, Esmail GA, Ghilan AKM, et al. (2020) Isolation and purification of starch hydrolyzing amylase from *Streptomyces* sp. Al-Dhabi-46 obtained from the Jazan region of Saudi Arabia with industrial applications. *J King Saud Univ* 32: 1226–1232. https://doi.org/10.1016/j.jksus.2019.11.018
84. Ertan F, Balkan B, Balkan S, et al. (2006) Solid state fermentation for the production of α-amylase from *Penicillium chrysogenum* using mixed agricultural by-products as substrate. *Biologia* 61: 657–661. https://doi.org/10.2478/s11756-006-0137-2
85. Kizhakedathil MPJ, Subathra Devi C (2021) Acid stable α-amylase from *Pseudomonas balearica* VITPS19—Production, purification and characterization. *Biotechnol Rep* 30: e00603. https://doi.org/10.1016/j.btre. 2021.e00603
86. Ahmed SA, Mostafa FA, Helmy WA, et al. (2017) Improvement of bacterial α-amylase production and application using two steps statistical factorial design. *Biocatal Agric Biotechnol* 10: 224–
233. https://doi.org/10.1016/j.bcab.2017.03.004

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Title: α-Amylase Study in *Geotrichum candidum*: Optimization of production, Purification and Enzyme Characterization

Abstract: Enzymes, as biocatalysts, are highly valued for their industrial applications. Among the various sources available, microbial enzymes, and in particular those from yeast, are of great interest due to their polyvalence and numerous biotechnological applications. This study aims to investigate the production of enzymes with high biotechnological potential from a local yeast strain. After isolation from various samples collected in different regions, 100 strains were grown and evaluated for their enzymatic capacity on substrates such as soluble starch, carboxymethylcellulose, Tweens and olive oil, using rapid plate detection methods at 28°C on YPGA and YMA media. Yeast PO27, selected for its significant α-amylase production as well as its lipolytic and esterolytic activities, and was identified as *Geotrichum candidum* by analysis of the D1/D2 region of the 26S ribosomal RNA sequence. To address the economic challenge associated with amylase production, this study focused on the use of inexpensive substrates such as olive pomace, potato peelings, leftover bread and mastic waste for solid-state and submerged fermentation. The results showed that olive pomace was the best substrate, achieving a maximum α-amylase production of 180.71 U/g through solid-state fermentation. Optimizing culture conditions with a central composite design, production was increased to 412.94 U/g under specific conditions of humidity, malt extract and $CaCl_2$. Physicochemical characterization of purified α-amylase revealed optimal activity at pH 5 and 70°C, with remarkable stability in the presence of Mg^{2+} and Tween 80, and resistance to surfactants and organic solvents. The enzyme showed notable efficacy in cotton desizing at room temperature, demonstrating its potential for low-cost, efficient industrial applications.

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