PECTINASE PRODUCTION FROM WILD STRAINS OF *ASPERGILLUS* SPP. ISOLATED FROM NATURAL RESOURCES

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ABSTRACT

PECTINASE PRODUCTION FROM WILD STRAINS OF *ASPERGILLUS* SPP. OBTAINED FROM NATURAL RESOURCES

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The increasing global fruit product consumption necessitates research for efficient juice extraction processes, where enzymes, particularly pectinases, play a crucial role. Pectinases can break down pectins in plant cell walls, enhancing extraction efficiency.

This study aims to investigate pectinase production from wild *Aspergillus* spp. strains isolated from natural resources in Ankara, Turkey. Additionally, screening for wild strains can provide a broader range of working conditions suited to different extraction processes. The focus of this research was also to obtain pectinase-producing strains with optimal working conditions for acidic environments and high temperatures commonly encountered in fruit juice extraction.

The proposed methodology involved the isolation and screening of *Aspergillus* spp. strains from environmental samples, followed by morphological analysis and single colony isolation. The isolated strains underwent pectinase assay using modified growth media. The strain exhibiting the highest enzyme activity (4.08 IU/mL) was further identified as *Aspergillus brasiliensis* and subjected to fermentation experiments to determine optimal nitrogen source, time and temperature.

The findings of this study are expected to contribute to the identification of pectinase-producing *Aspergillus* spp. strains with favorable enzymatic activity under acidic and high-temperature conditions (pH 5.5, 50° C). Ultimately, the chosen isolate exhibited high enzyme activity (9.99 IU/ml at 120 hour) and specific activity (148.42 IU/mg at 48 hour) at 30°C with multiple nitrogen sources. The utilization of wild strains and the exploration of their enzymatic potential have the potential to drive innovation and advancements in various biotechnological fields.

Keywords: *Aspergillus*, pectinase, fruit juice, wild strain, isolation

ÖZ

DOĞAL KAYNAKLARDAN İZOLE EDİLEN YABANİ *ASPERGİLLUS* TÜRLERİ İLE PEKTİNAZ ÜRETİMİ

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Küresel meyve ürünü tüketiminin artması, verimli meyve suyu ekstraksiyon süreçleri için araştırmaların yapılmasını gerektirir. Bu süreçte, özellikle pektinazlar olmak üzere enzimlerin önemli bir rolü vardır. Pektinazlar bitki hücre duvarlarındaki pektinleri parçalayarak ekstraksiyon verimliliğini artırabilir.

Bu çalışma, Ankara, Türkiye'deki doğal kaynaklardan izole edilen yabani *Aspergillus* spp. suşlarından pektinaz üretimini araştırmayı amaçlamaktadır. Ayrıca, yabani suşların taranması, farklı ekstraksiyon süreçlerine uygun daha geniş çalışma koşulları sağlayabilir. Bu araştırmanın odak noktası, meyve suyu ekstraksiyon işlemlerinde yaygın olarak karşılaşılan asidik ortamlar ve yüksek sıcaklıklar için optimal çalışma koşullarına sahip pektinaz üreten suşları elde etmektir.

Önerilen metodoloji, çevresel örneklerden *Aspergillus* spp. suşlarının izolasyonu ve taramasını, ardından morfolojik analiz ve tek koloni izolasyonunu içermektedir. İzole edilen suşlar, değiştirilmiş büyüme ortamı kullanılarak pektinaz analizine tabi

tutulmuştur. En yüksek enzim aktivitesini gösteren suş (4.08 IU/mL), daha sonra *Aspergillus brasiliensis* olarak tanımlanmış ve optimal azot kaynağı, zaman ve sıcaklık belirlemek için fermantasyon deneylerine tabi tutulmuştur.

Bu çalışmanın bulgularının, asidik ve yüksek sıcaklık koşullarına (pH 5.5, 50°C) elverişli, yüksek enzimatik aktiviteye sahip pektinaz üreten *Aspergillus* spp. suşlarının belirlenmesine katkıda bulunması beklenmektedir. Sonuç olarak, seçilen izolat, 30°C'de çoklu azot kaynaklarıyla, yüksek enzim aktivitesi (120 saatte 9.99 IU/ml) ve spesifik aktivite (48 saatte 148.42 IU/mg) sergilemiştir. Yabani suşların kullanımı ve enzimatik potansiyellerinin keşfi, çeşitli biyoteknoloji alanlarında yenilik ve ilerlemelerin öncüsü olacaktır.

Anahtar Kelimeler: *Aspergillus*, pektinaz, meyve suyu, yabani suş, izolasyon

Dedicated to all those who seek knowledge, challenge boundaries, and dare to dream…

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CHAPTER 1

INTRODUCTION

In recent years, humankind faced a lot of natural disasters and a pandemic, which halted the daily life of billions of people. In the face of such crises, most of the world population was sheltered in their own houses to save their health and avoid spread of disease while scientists, health care workers, and essential workers tried to stabilize and sort out the problems.

During these crises, the food consumption trend of the world population started to change and many consumers began to consume a lot of fruit products (e.g. fruit juices, molasses, jams etc.), as a healthy option to prevent the occurrence of diseases (Autentika Global, 2021; Kumar & Babu, 2021). However, it is important to consume those in moderation and as part of a balanced diet. There are many criteria to consider when consuming a product as a part of a balanced diet. First, it should not contain added sugars or artificial flavors, and it should not exceed daily intake limits.

According to the article published by The Center for Disease Control and Prevention (*Only 1 in 10 Adults Get Enough Fruits or Vegetables | DNPAO | CDC*, n.d.), the current recommended daily intake limit for fruit juices is given as 473 ml. Similarly, Ruxton and Myers (2021) stated that health benefits of fruit juice consumption are weighing up potential health risks even with up to 500 ml per day. In this study, authors reviewed many studies that concern the effects of fruit juice consumption on human health, and concluded that risks like obesity, type 2 diabetes, cardiovascular

disease or poor glycemic control do not increase, furthermore, adequate juice consumption also decreases vascular problems and blood pressure.

As the trend shifts toward a healthy diet, production of fruit juices and molasses also increased in response to increased consumption of fruit juices and molasses. The most crucial bottleneck in the production of fruit juice and molasses is the efficiency of juice extraction processes (Antonio Bizzo et al., 2014). In order to increase the efficiency, many physical techniques are applied, yet the most effective way to increase efficiency arguably is the use of enzymes that accelerate the process and increase the yield of extraction.

Eduard Buchner used enzymes for the first time in 1833; discovering yeast extracts, devoid of living cells, to convert sugar into alcohol. However, William Kühne was the first person to use the term “enzyme” in 1877(Heckmann & Paradisi, 2020). These advancements led to first industrial enzyme production that is diastase.

In the mid-20th century, implementation of enzymes in the food industry expanded to new fields, which made production easier. For instance, cheese and bread makers, and brewers were extensively using this technology to increase their production. During the late 20th century, researchers further studied enzymes, along with other bio-chemicals, and their interactions triggered other industries to adopt this technology in their production.

Nowadays, enzymes are used extensively by various industries (food, textile, paper, pharmaceutical, biotechnology etc.) due to their specificity, efficiency, and sustainability. For instance, in the food industry, enzymes are used to improve the texture, flavor, and nutritional value of foods, whereas, they are used in processing

to achieve various effects such as bio polishing, de-sizing, and stonewashing in the textile industry. In the paper industry, enzymes are used to reduce the environmental impact of paper manufacturing and improve the quality of paper, they are utilized in the production of various drugs and therapies in the pharmaceutical industry. Moreover, enzymes are also used extensively in the biotechnology field for various applications such as DNA sequencing, gene expression analysis, and protein production.

Enzymes are used in industry, not only for providing efficiency and sustainability, but also for creating new products, saving energy and time and generating new pathways for time-consuming long processes and applications. Some common examples of these are conversion of substances into other substances (e.g. amylases are utilized to convert starch to simple sugars), breakdown of unwanted components (e.g. lactases are used to breakdown lactose in dairy products, xylanases are used to decompose hemicellulose in paper industry), shelf life extensions of products (e.g. maltogenic amylases are used to improve shelf life of baked products), production of important components (e.g. proteases are used to produce insulin), and research and development of new technologies in biotechnology field (e.g. DNA amplification and cloning).

In summary, enzymes are used for a wide range of applications, including food processing, textile processing, paper manufacturing, pharmaceuticals, and biotechnology. Enzyme use has many advantages, such as improved efficiency, reduced environmental impact, and increased sustainability. Many of these enzymes became an essential part of different processes with their unique characteristics.

In the fruit juice industry, varieties of enzymes are utilized for different purposes, including juice extraction, clarification, and flavor development. While enzymes like

xylanases, cellulases, hemicellulases and pectinases are used to increase the efficiency of the extraction process, enzymes such as amylases and glucose oxidases are utilized to convert fermentable components to unfermentable components. On the other hand, proteases, lipases and catalases are generally used to slow down/stop off-flavor formation.

Each of the enzymes that increases the yield has different effects on the fruit juices. For instance, cellulases, glycosyl hydrolases, are added to hydrolase cellulose in cell walls of plant cells, while xylanases are added to hydrolase xylan in cell walls. Both of these enzymes are used to increase yield of extraction by decomposing cell walls. Pectinases are used to break down pectins in between cell walls by means of hydrolysis or trans-elimination or deesterification, depending on their types. Pectinases not only increase fruit juice yield, but also clear the juice, decrease viscosity of the fluid and increase flow rate of juice through filters.

Among the aforementioned enzymes, pectinases are utilized in many industries, including food, textile, paper, and pharmaceuticals, because of their ability to break down pectins, a heteropolysaccharide commonly found in plant cell walls. There are three main types of pectinases, found naturally, that break down pectins. Pectin esterases deesterify the methoxyl group of pectins to form pectic acid, hydrolases cleave α-1,4-glycosidic linkage of pectic acids and pectins, whereas, lyases breakdown α-1,4-glycosidic linkage of pectic acids and pectins by trans-elimination and forms unsaturated galacturonates and methyl galacturonates.

In nature, pectinases are produced by various species (e.g. fungi, mold, bacteria and plants etc.). Most common sources of industrial pectinases are from molds and bacteria. In industrial applications, criteria like specific and enzymatic activity, optimum temperature and pH of the medium that is going to be treated are among

the reasons for the searching and engineering of better enzymes. Depending on the application, enzymes that work better in acidic conditions or enzymes that can withstand high temperatures are highly sought.

Aspergillus spp. are commonly used for the production of enzymes, since enzymes produced are mostly exo-enzymes and have high enzymatic activity. However, mold species are arduous to work on both lab and industrial production scale due to formation of spores that can contaminate surfaces and air. For this reason, molds are generally avoided. However, many different sub species of *Aspergillus* were already used to produce pectinase enzymes (Haile & Ayele, 2022; Khatri et al., 2015).

On another note, research studies related to enzyme production mainly rely on non-commercial strains and the use of these strains are mostly restricted. Studies carried out in research and development departments in industry cannot commercialize research products that are produced by/from non-commercial components. Thus, it is crucial for industrial producers to work with wild strains. However, isolation and characterization of wild strains and/or getting permissions to use these microorganisms are a long and tedious process. One must also consider all of the possible hazards that these microorganisms may cause in laboratories, factories, researchers and workers.

On the other hand, isolation of wild strains is beneficial not only to producers, but also to researchers in different fields that may use these strains for their own research purposes. Since the isolation from local environmental sources (e.g. soil, tree stumps, hot springs, rivers etc.) will give researchers endemic wild types of strains that are prevalent in current geography, this may result in finding new subspecies of sought microorganisms and result in more studies that may help with breakthroughs in current technology.

While screening for wild strains that can produce enzymes with better optimum working conditions suited to processes (i.e. temperature, pH, stability, etc.), all the strains isolated may provide a wider range of working conditions for the processes. This is why all isolated strains will be an asset to researchers in the end. Moreover, one type of species may produce more than one type of product and this may open new research areas for the research and development field.

In fruit juice and molasses production, juices are usually acidic due to the nature of fruits that they are extracted from, however, optimum temperature for the enzyme always depends on the processes of extraction. Due to time constraints and efficiency issues, extraction processes are mostly carried out simultaneously (e.g. heat-assisted extraction with tumbling). Such processes also contain enzyme treatment that can withstand current physical conditions present in the extraction medium.

Enzymes that are produced by species have different characteristics depending on the habitat in which producer species thrive. This is true for all species including humans. Enzymes produced by humans work best in usual body temperature (Duggan, 1979), it is also same for pathogens that infect humans. However, *Aspergillus* spp. can thrive under many different environments (e.g. plants, soil, air, animal systems, and waterbodies), because they have high stress tolerance and adaptation abilities due to being able to use vast amount of organic substances (Paulussen et al., 2017).

In this study, production of pectinase enzymes with optimum conditions specific to the processes in the fruit juice industry was carried out using *Aspergillus* species, which is a common pectinase producing molds, isolated from environmental samples (soil, tree stumps, hot springs, rivers etc.). Since the juices and molasses treated will

be mostly acidic and current extraction methods generally require high operation temperatures, it is expected that produced pectinase enzymes should have affinity to work better in acidic conditions and high temperatures.

Isolation process included cell wall repair stage, selective enrichment stage, morphological analysis and single colony isolation. After this stage, isolates were frozen and stored. Later on, frozen isolates were screened for pectinase enzymes using modified growth media and enzyme activity analysis. Confirmed pectinase producing mold with the highest enzyme activity was serotyped and fermentation medium and physical conditions for fermentation were tested to obtain better enzyme activities. Since this study consisted of a wild *Aspergillus* spp., optimization process was not carried out, but independent variables were tested for their effect on enzyme activity.

CHAPTER 2

LITERATURE REVIEW

2.1 Pectin and Its Use

Pectin is a general name given to heteropolysaccharide commonly found in fruits. This carbohydrate was firstly announced as hemostatic agent in 1935, and claimed to decrease bleeding (Verstraete, 1977).

Pectin is present in all plants, and each plant may produce it in a different way to utilize its diverse functional properties. Its most known property is its gelling ability, which is known for centuries (Bemiller, 1986). Pectin is a complex macromolecule, composed of plant based galacturonans, and most of these are linear chain of (1→4)-linked α-D-galactopyranosyluronic acid units.

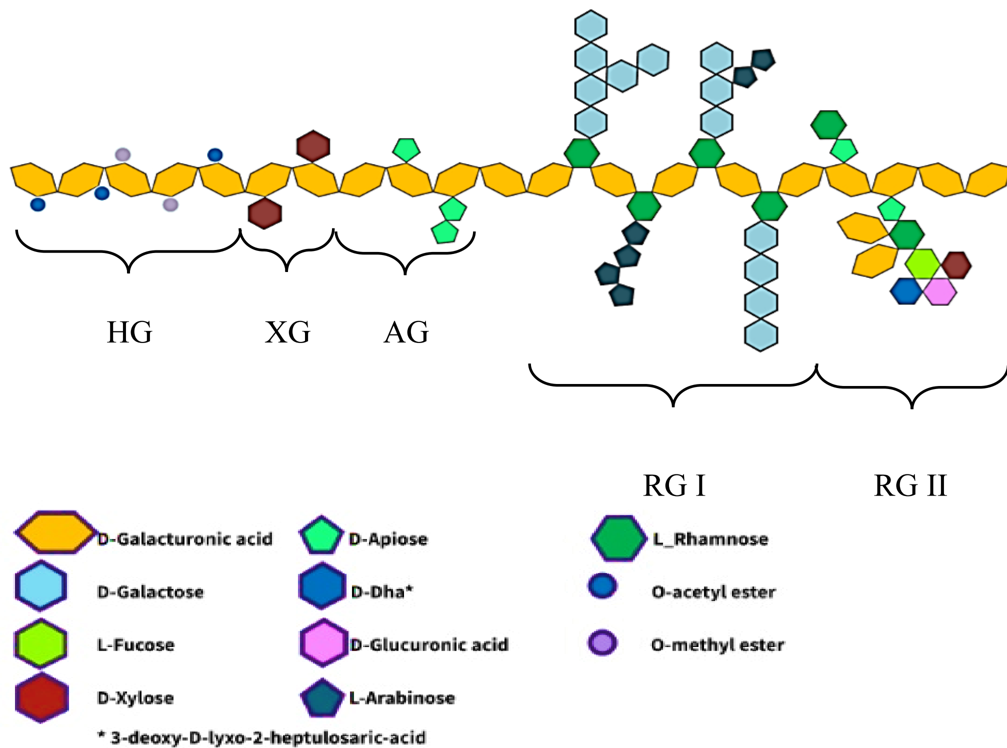

Figure 1. Pectin structures

Pectin produced by plants has four common chain structures, and most commonly seen structure (about 60 percent) is Homogalacturonan (HG) that consists of polygalacturonic acid chain. Another chain structure is Xylogalacturonan (XG) that has polygalacturonic acid chain as backbone and xylose in branches. Last two chain structures are Rhamnogalacturonan I (RG I) and Rhamnogalacturonan II (RG II). RG I is made of backbone of alternating galacturonic acid and L-rhamnose and polymers of L-arabinose and D-galactose attached to L-rhamnose. RG II contains polygalacturonic acid chain as backbone with side chains of many complex glycan, comprising many varieties of neutral sugars, attached to it (Fig.1.).

Pectin has many uses in industrial applications, including food, pharmaceutical, cosmetic, textile, paper and biomedical industries. In food industry, it is used as a gelling agent in various food products like jams, jellies and other fruit-based products and as a thickener to increase viscosity in beverages, dairy products, and sauces. While it is used to create controlled-release drugs and used as a binder in tablet formulations in pharmaceutical industry, it is used in drug delivery systems and tissue engineering in biomedical industry. In addition, it is utilized in cosmetics industry as a thickener and stabilizer, and in textile industry, to create natural dyes and thicken printing pastes. Lastly, in paper industry, it is employed as a paper coating binder and as strengthening and smoothing agent for papers.

Even though there are many uses of pectin in various industries, in some cases pectin is not wanted because of its unique chemical and physical properties. One such example is fruit juice production. In the extraction process, pectin between fruit cell walls decreases the efficiency of the extraction. Moreover, pectin, since it causes gelling in juice during production, may cause clogging in the system and inconsistency in the juice.

In order to avoid problems arising from presence of pectin, industrial applications use several of physical, chemical and biological technique for decomposition or filtration of pectin, naturally present in fruits. Among all these techniques, most common and arguably most effective technique is the use of enzymes to decompose pectin.

2.2 Pectinase

Pectinase enzymes are involved in the breakdown of pectin, a complex heteropolysaccharide found in plant cell walls. Pectin is a major component of the middle lamella that holds plant cells together, and it plays a crucial role in the structure and function of plant tissues. Pectin is composed of a complex network of galacturonic acid units that are linked together by α-1,4-glycosidic bonds(Chen et al., 2021). The pectin network is stabilized by various cross-linking agents, including calcium ions, borate ions, and covalent bonds.

Pectinase enzymes have a wide range of applications in various industries. In the food industry, pectinases are used to improve the texture of fruits and vegetables, to clarify fruit juices, and to enhance the extraction of flavors and colors from plant materials. They are also used in the production of pectin, a natural gelling agent used in the production of jams, jellies, and other food products.

These enzymes are generally produced by a wide range of microorganisms and can be found in some plant tissues. Most common source of many types of enzymes are microorganisms (mold, yeast and bacteria). Microorganisms that can metabolize fruits generally have pectinases; however, environmental conditions like temperature or acidity, to which the microorganisms are subjected, show similarities with the optimum working conditions of their enzymes. In other words, isolates obtained from thermal sources most probably have enzymes that can work in high

temperatures, while isolates obtained from alkaline environment have enzymes that work best in alkaline environments.

Pectinase enzymes are proteins with complex tertiary structures crucial for their functions, and coded in the genes. The sequence of amino acids in pectinase enzymes differs depending on the organism and type of enzyme, but they all have important amino acids that are crucial for their function. These amino acids are located in the active site of the enzyme and interact with the substrate during the reaction.

Pectinase enzymes catalyze the hydrolysis of the glycosidic bonds in pectin, which leads to the breakdown of the pectin network. Pectinase enzymes are grouped under three main categories. First category of pectinases are pectin esterases and they form pectic acid from pectin by de-esterifying the methoxyl groups. Second group of enzymes are hydrolases that cleave α-1,4-glycosidic linkage of pectic acids and pectin. Last group is pectin lyases that breakdown α-1,4-glycosidic linkage of pectic acids and pectins by trans-elimination and forms unsaturated galacturonates and methyl galacturonates.

In general, pectinase enzymes are composed of one or more domains that have distinct functions. For example, polygalacturonases, which are the most common type of pectinase, are composed of a catalytic domain and a carbohydrate-binding module (CBM)(Osete-Alcaraz et al., 2020). The catalytic domain is responsible for the hydrolysis of pectin, while the CBM binds to the pectin substrate and enhances the catalytic activity of the enzyme.

Pectin lyases, on the other hand, do not have a CBM domain, and their catalytic domain is composed of a series of β-strands that form a β-helix structure(Zheng et

al., 2021). This fold is conserved among all pectin lyases and is essential for their catalytic activity.

2.3 Aspergillus

Aspergillus is the name of a genus of ubiquitous filamentous fungi that contains more than 300 species and belongs to the phylum Ascomycota, order Eurotiales, and family Aspergillaceae.(Fang & Latgé, 2018). The fungus has a vegetative mycelium that consists of hyphae, which are multicellular structures that grow by apical extension and branching. The hyphae can be septate or aseptate, depending on the species. The mycelium produces asexual spores called conidia, which are formed at the tips of specialized structures called conidiophores (Riquelme et al., 2018). The conidia are small, airborne, and can be inhaled by humans and animals. Most of these species are saprophytes and opportunistic pathogens in humans and animals.

The conidia of *Aspergillus* have a characteristic morphology that allows them to be easily recognized under the microscope. They are typically oval or spherical, with a smooth or rough surface, and are often covered with a layer of hydrophobic proteins that protect them from desiccation. The conidia are dispersed by air currents and can remain viable for long periods under favorable conditions, such as low humidity and high temperature. When the conidia land on a suitable substrate, they germinate and form new mycelia, which can produce more conidia.

The life cycle of *Aspergillus* is complex and involves sexual and asexual reproduction. The sexual cycle of *Aspergillus* is not well understood, and only a few species have been observed to undergo sexual reproduction in the laboratory. The asexual cycle of *Aspergillus*, on the other hand, is well documented and is responsible for the production of the vast majority of conidia. The asexual cycle involves the formation of conidiophores, which are specialized structures that

produce conidia by mitosis. The conidiophores are formed from hyphae that differentiate into specialized cells called phialides, which are responsible for the production of the conidia. The conidia are formed by a process called blastic conidiation, in which a bud-like structure emerges from the phialide and grows into a mature conidium.

The most common species of *Aspergillus* includes *A.* fumigatus, *A.* flavus, *A.* niger, *A.* terreus, and *A.* nidulans. These fungi are commonly found in soil, decaying vegetation and animal carcasses, and animal feces and they can cause a range of diseases, including allergies, asthma, aspergillosis, and mycotoxicosis. Aspergillosis is a group of diseases caused by *Aspergillus*, which affect different organs and tissues in humans and animals, depending on the species and the immune status of the host. Aspergillosis is a significant public health problem worldwide, particularly in immunocompromised individuals, such as transplant recipients, cancer patients, and HIV-infected individuals.

The taxonomy of *Aspergillus* has gone through significant changes in recent years, with the development of molecular techniques that have enabled the identification of new species and the reclassification of existing ones (Tsang et al., 2018). The genus *Aspergillus* is currently divided into six subgenera, including *Aspergillus*, Circumdati, Fumigati, Cremei, Nidulantes, and Polypaecilum (Houbraken et al., 2020). Each subgenus contains numerous species that are distinguished by morphological, physiological, and molecular characteristics.

The taxonomy studies of *Aspergillus* are crucial for numerous reasons. First, it provides a base for researchers for classification and identification. This is important for researchers since it helps them to see and study the evolutionary relationship between species. Moreover, it helps for the diagnosis of diseases and treatment of aspergillosis. Correct diagnosis of the aspergillosis lowers the time to respond to the

disease with correct treatment methods. In addition to this, it helps researchers to study the ecology and distribution of *Aspergillus* species. Lastly, it can provide researchers to choose appropriate species that study and use these species to produce several metabolites (enzymes, chemical compounds, biofuels, etc.) via fermentation.

Aspergillus species causes several types of diseases, and these diseases are differentiated depending on site of infection and the immune status of the host. Some common types of aspergillosis are allergic bronchopulmonary aspergillosis (ABPA), allergic *Aspergillus* sinusitis, Azole-Resistant *Aspergillus* fumigatus, aspergilloma, chronic pulmonary aspergillosis, invasive aspergillosis and cutaneous (skin) aspergillosis (*About Aspergillosis | Aspergillosis | Types of Fungal Diseases | Fungal Diseases | CDC*, n.d.).

In order to diagnose *Aspergillus* infections, a combination of clinical, radiological, and microbiological criteria is required. The clinical presentation of aspergillosis is nonspecific, and the symptoms can vary depending on the host, the type and severity of the infection. Radiological imaging, such as chest X-rays or CT scans, can help to identify the characteristic features of aspergillosis, such as pulmonary infiltrates, nodules, or cavities (*Diagnosis and Testing for Aspergillosis | Aspergillosis | Types of Fungal Diseases | Fungal Diseases | CDC*, n.d.). Microbiological diagnosis involves the isolation and identification of *Aspergillus* from clinical specimens. The identification of *Aspergillus* is usually based on the morphology of the fungal colonies and the production of characteristic spores, such as conidia or sclerotia. Molecular techniques, such as PCR and DNA sequencing, should be used to identify *Aspergillus* species and detect drug resistant mutants.

On the other hand, like many mold types, *Aspergillus* species are used thoroughly in the industry for variety of metabolites, they produce. Especially, they are highly

sought for the production of several enzymes, such as pectinase, amyloglucosidase, cellulase, β-glucanase and lipase. Main reason for the selection of *Aspergillus* species for the production of enzymes is that *Aspergillus* species have high secretion ability (Ntana et al., 2020), and their enzymes have higher enzyme activity and stability (thermostable, wide pH range, more stable in organic solvents, and enantioselectivity toward the substrate) compared to other species (Contesini et al., 2016).

2.4 Pectinase Production by *Aspergillus*

Numerous microorganisms are capable of producing pectinases, but *Aspergillus* species have gained significant attention due to their remarkable enzyme production capabilities.

Various fungal species, such as *Trichoderma* reesei, *Penicillium* spp., and *Fusarium* spp., have been explored for pectinase production (Ferreira et al., 2010; Schneider et al., 2016). However, compared to *Aspergillus* species, these fungi often exhibit lower enzyme yields and limited substrate specificity. *Aspergillus* species, with their superior enzyme production capabilities, outperform many other fungi in terms of pectinase production.

Bacterial strains, including *Bacillus* subtilis and *Pseudomonas* fluorescens (Basheer et al., 2021; Jayani et al., 2010), have also been studied for pectinase production. Although these bacteria can produce pectinolytic enzymes, they often require complex media formulations and genetic modifications to achieve enzyme yields comparable to *Aspergillus* species. Additionally, the diversity and specificity of

pectinolytic enzymes produced by bacteria are usually more limited compared to fungi.

Aspergillus species are recognized for their ability to produce enzymes with high activities and stability. *Aspergillus* species naturally uses various enzymes to degrade certain compounds present in the environment to proliferate by using the decomposed substances. Pectinases are among the enzymes that is used by *Aspergillus* species to decompose pectin present in plant cell walls.

Aspergillus niger and *Aspergillus flavus* are most known producers of pectinase among the species of *Aspergillus*. The reason for these fungi known as best producers among other species is that they possess the necessary metabolic pathways and regulatory mechanisms to efficiently synthesize and secrete pectinase enzymes. When comparing the pectinase production and characteristics among different *Aspergillus* species, variations in enzyme yield, substrate specificity, and optimal conditions can be observed. For instance, studies have shown that *A.* niger exhibits high production yields of pectinase enzymes and a broad substrate specificity, making it suitable for various applications (Liu & Kokare, 2023; Rosales et al., 2018; Yadav et al., 2023). *A. flavus*, on the other hand, may produce pectinases with distinct characteristics and substrate specificities, which may influence its applicability in specific industries or processes (Pařenicová, 2000).

The production of a metabolite by a microorganism is affected by various factors. In the same way, the production of pectinase enzymes by *Aspergillus* species can be influenced by various factors, including culture conditions, carbon and nitrogen sources, pH, temperature, and inducers. Different *Aspergillus* species may display deviations in the production yield and kinetics of pectinase enzymes, which can influence their practical applications.

Aspergillus species produces various types of pectinases and these enzymes are classified into different categories based on their specific activities and substrate specificities. These include polygalacturonases, pectin lyases, and pectate lyases. The specific characteristics of pectinase enzymes, such as optimal pH and temperature, stability, and substrate affinity, may differ among different *Aspergillus* species. Understanding these characteristics is essential for improving enzyme production and simplifying their applications in different industries.

Pectinase enzymes produced by different *Aspergillus* species exhibit variations in production yields, characteristics, and applications. *Aspergillus* species, particularly *A. niger*, are known for their ability to produce large quantities of pectinases. Their robust growth and metabolism allow for high enzyme production, resulting in cost-effective industrial processes. Understanding these differences is crucial for selecting the most suitable *Aspergillus* species and optimizing enzyme production processes for specific industrial applications. Further research and biotechnological advancements in the field of pectinase production and engineering can unlock the full potential of these enzymes,

Aspergillus species can be genetically manipulated to enhance enzyme production. Techniques such as strain improvement, gene overexpression, and metabolic engineering have been successfully employed to further increase pectinase yields, expanding the potential of *Aspergillus* as a microbial cell factory. Advances in omics technologies, such as genomics and transcriptomics, have provided insights into the molecular mechanisms underlying pectinase production (de Vries et al., 2017; Martens-Uzunova, 2008). Furthermore, the discovery and characterization of novel pectinolytic enzymes from *Aspergillus* and other microorganisms have opened new avenues for tailored enzymatic applications.

2.5 Submerged Fermentation

Submerged fermentation, also known as liquid-state fermentation, has developed as a highly favorable method for pectinase production. This technique involves the growth of microorganisms and enzyme production in a liquid medium. There are numerous advantages offered by submerged fermentation. It provides enhanced environmental control, process scalability, simplified process monitoring, efficient nutrient utilization and minimized process variability.

It enables precise control of crucial environmental parameters, including temperature, pH, dissolved oxygen, and nutrient availability. These optimized conditions promote superior microbial growth and enzyme production, which results in higher yields.

The liquid medium used in submerged fermentation provides a larger surface area for microbial growth, leading to increased enzyme production. Moreover, the use of liquid medium provides the ability to easily scale up, which is crucial when large-scale production is desired for industrial applications.

Furthermore, real-time observation of the submerged fermentation process allows sampling the liquid medium for regular measurements of microbial growth and enzyme activity, providing actual insights into the fermentation process.

In submerged fermentation, nutrients efficiently utilized by microorganisms for growth and enzyme production since they have direct access to nutrients in the liquid medium. This efficiency contributes to higher productivity and cost-effectiveness.

Submerged fermentation offers reduced process variability in comparison to solid state fermentation. The controlled conditions in the liquid medium minimize the influence of external factors, leading to consistent and predictable enzyme production.

Submerged fermentation technique provides precise control over environmental conditions, enables efficient nutrient utilization, simplifies process monitoring, and ensures scalability (Fig.2.). The advantages offered by submerged fermentation make it an attractive choice for industrial applications requiring high-quality pectinases.

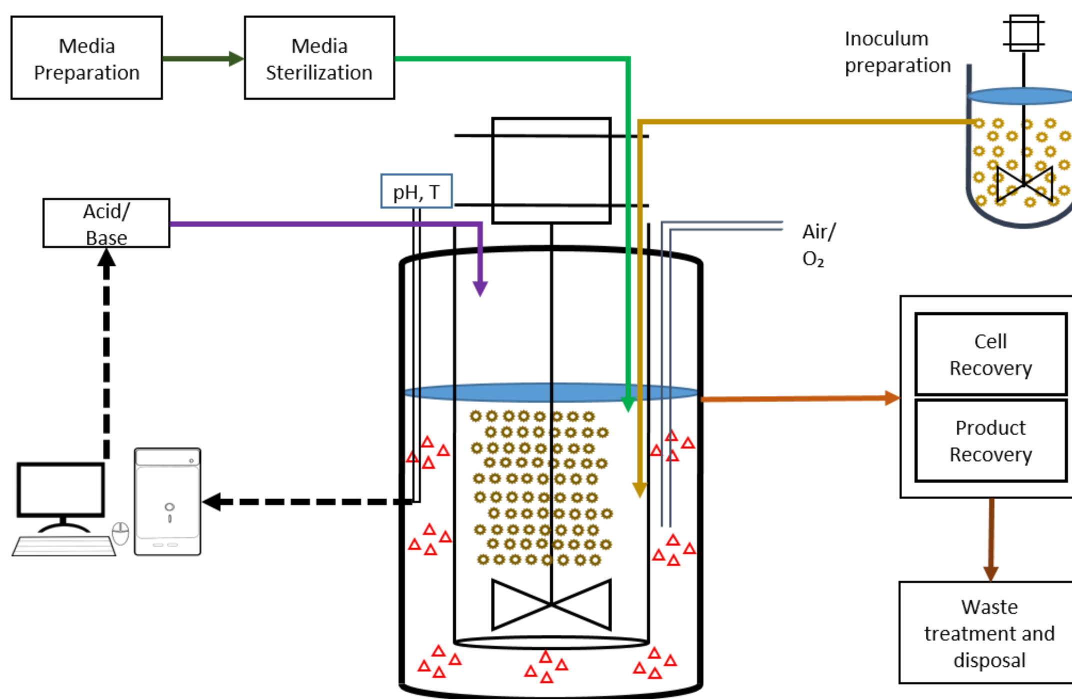

Figure 2. Submerged Fermentation Diagram

2.6 Aim of The Study

In this study, it was aimed to isolate a wild *Aspergillus* species that is a capable pectinase enzyme producer, which can be used in industrial fermentation processes. For this reason, *Aspergillus* spp. were meticulously isolated from various environmental samples collected in Ankara, encompassing a diverse range of habitats such as soil, tree trunks, and water bodies. Selective media were used to differentiate species from each other and one colony forming unit was transferred to a new medium to further isolate species from the other species. Isolated species were then transferred to cryotubes to store in freezers. After that isolates were identified by conventional methods. Later on, identified isolates were screened for pectinase production capacities in various media to compare enzyme activities and specific activities with each other to choose the better pectinase producer. Finally, various media for enzyme production were tested for higher activities. This stage consisted of determination of best medium composition, pH and incubation time for highest enzyme activity.

CHAPTER 3

MATERIALS AND METHODS

3.1 Strains

Strains used in the study were obtained from environmental sources (i.e. tree stumps, surfaces and soils) from Ankara, Turkey. For surfaces and tree stumps, sterile cotton swabs were rubbed to sample surface of 0.01 m^2 and dipped into screw capped tubes containing 10 ml of Buffered Peptone Water (BPW). On the other hand, solid samples were collected with sterilized utensils and 25 g of sample is weighed into a sterile filter bag, containing 225 ml of BPW. After homogenization of the samples in BPW, samples were incubated in 28° C for 16 hours to allow microorganisms to repair their cell wall. At the end of incubation, 1 ml from each sample was taken and spread onto Rose Bengal Chloramphenicol Agars (RBC) using glass triangles. RBC agars are a selective medium that contains antimicrobial chloramphenicol, which prevents the growth of bacteria. They also provide differentiation for the molds and yeasts. In this medium, *Aspergillus* species form black colonies with white mycelium. Inoculated agars, then, incubated for 48 hours at 28° C for microorganisms to form colonies. After 48 hours, black colonies with white mycelium were suspected to be *Aspergillus* species. One suspected colony from each agar was taken to a fresh RBC agar using an inoculation loop and incubated to fresh RBC Agar for another 48 hours at 28° C for pure colony formation. Then, one black colony was chosen from the new agar and transferred to Potato Dextrose Broth (PDB) for 48 hours at 28° C. Later on, 0.15 ml of glycerol and 0.85 ml of previously prepared culture were pipetted into 2 ml cryotubes and stored at -30° C.

Each frozen strains were screened for pectinase production and those who could produce pectinase were sent to Düzen Norwest Laboratory to identify the species of isolates. Düzen Laboratory used microscopic examination to determine and differentiate the morphological features and slide culture technique to stain and identify fungal structures for identification of *Aspergillus* strains. Identified colonies were labeled and replicated for storage.

3.2 Initial Pectinase Production Medium

Initial medium used for pectinase production was modified version of various studies (Abdullah et al., 2018; El Enshasy et al., 2018). It consisted of pectin, 30g/L; (NH4)2SO4, 3.33g/L; K2HPO4, 0.5g/L; MgSO4.5H2O, 0.05g/L in 100 ml of distilled water. After dissolving solids in water, pH was set to 5.5 using citric acid and sodium hydroxide, and autoclaved at 121° C for 20 min to sterilize the solution. The sterilized solution was then stored in refrigerator at 4° C until use.

3.3 Pectinase Production with Submerged Fermentation

Ten milliliter of PDB was inoculated with isolate and incubated at 30° C for 48 hours. Then, the method described by Petrikkou and coworkers (2001) was carried out to measure the cell density of the growth medium in spectrophotometer at 530 nm (OD_{530}). Samples were either diluted or kept in incubator until 0.5 was read in spectrophotometer at OD_{530} to obtain similar cell densities (1.0 × 106 and 5.0 × 106 CFU/ml). After incubation, 10 ml of inoculum in PDB was pipetted into 1 liter of fresh pectinase production medium and incubated at 28° C for 3 days by using a magnetic stirrer set at 300 rpm. The pH of the fermentation medium was controlled at 6 hour intervals by using Hanna HI 2020 Edge® pH Meter, and it was adjusted to pH 5.5 using HCl and NaOH solutions.

At the end of the incubation period, the production medium was filtered first using mutton cloth and then using Whatman no 1. Filtered medium was then collected into

falcon tubes and centrifuged at 4000 rpm 25 minutes to remove mycelial mass and cell debris(Khatri et al., 2015b). After centrifuging, supernatants, exo-enzymes, in each falcon tube were collected and stored at 4° C (Fig.3.).

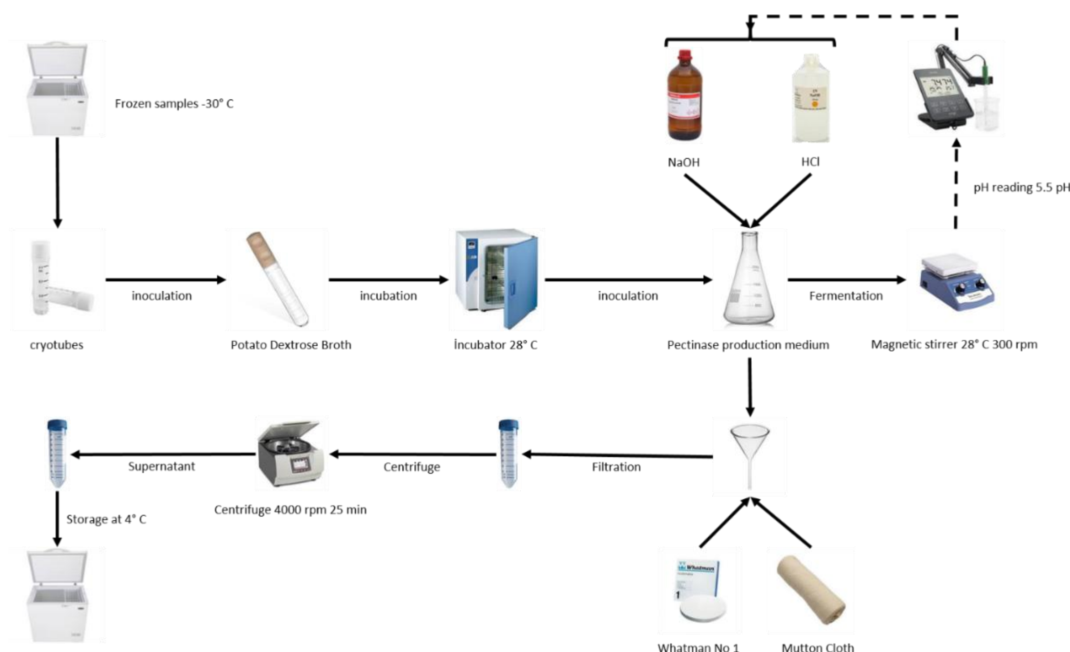

Figure 3. Pectinase production in lab scale

3.4 Enzyme activity

The 3,5-dinitrosalicylic acid (DNS) method described by Miller (1959) was used to determine pectinase activity. The DNS method is based on the detection of reducing groups produced during the enzymatic hydrolysis of the substrate. Exo-PG specifically acts on the polygalacturonic acid component of pectin, releasing galacturonic acid monomers. These monomers have reducing properties and react with the DNS reagent, resulting in the formation of a colored product. The intensity of the color formed is directly proportional to the concentration of reducing sugars, which can be quantified spectrophotometrically.

The measurement of exo-polygalacturonase (exo-PG) activity is based on the release of D-galacturonic acid from pectin during a defined period of reaction. One unit of

exo-PG activity is equivalent to the amount of enzyme that liberates 1 millimole (mmol) of D-galacturonic acid per minute (U = µmol min^{-1}) under the specified test conditions. To determine this activity, a standard curve was established using α-D-galacturonic acid as the reducing group, ranging from 0.1 to 10 milligrams per milliliter (mg mL^{-1}). The exo-PG activity was then expressed as activity units per milliliter (U mL^{-1}).

For the experiment, acetate buffer solution (0.05M) was prepared at pH 4.5 by using sodium acetate (Sigma-Aldrich, anhydrous, for molecular biology, ≥99%), acetic acid (Sigma-Aldrich, glacial, ACS reagent, ≥99.7%) and hydrochloric acid (Sigma-Aldrich, ACS reagent, 37%). Following that, 1% (w/v) solution of D-galacturonic acid (Sigma-Aldrich, ≥97%) and 1% (w/v) solution of pectin (Alfasol) (substrate solution) were prepared in acetate buffer solution.

In order to obtain the standard curve, 0.5 ml of D-galacturonic acid at different concentrations (0.0625, 0.125, 0.25, 0.5 and 1%) and 0.5 ml deionized water were mixed into different glass tubes. While 0.5 ml of substrate solution and 0.5 ml enzyme extract was added to glass tube for enzyme activity, 1 ml of deionized water was added to another glass tube for blank sample.

Glass tubes, then, were placed in a water bath at the desired temperature for 30 minutes. After the incubation period, 3 mL of DNS reagent was added to each reaction tube to stop the reaction, and the reaction tubes were placed in a boiling water bath for approximately 3 minutes to intensify the color. After the color development, the tubes were removed from the water bath and cooled to room temperature.

Each reaction mixture was transferred into separate cuvettes, and the absorbance of the solutions were measured at 550 nm using a spectrophotometer (Shimadzu UV-1900i). Finally, enzyme activity was calculated using following formula:

$$Enzyme\ Activity\ \left(\frac{\frac{IU}{ml}}{min}\right) = \frac{absorbance\ of\ enzyme\ soln \times standard\ factor}{time\ of\ incubation\ (\mathrm{min})}$$

Equation 1

$$Standard\ factor = \frac{Concentration\ of\ standard\ \left(\mu\frac{mol}{ml}\right)}{absorbance\ at\ 550nm}$$

Equation 2

3.5 Specific activity

Protein determination was carried out according to the method described by Bradford (1976). Bovine serum albumin (BSA) (Sigma-Aldrich, heat shock fraction, protease free, pH 7, ≥98%) was used as standard (250 μg/mL) at different concentrations (Table 1). Then, deionized water and Commassie Brilliant Blue G250 (Sigma-Aldrich, for electrophoresis, pH 6.4 (10 g/l, H_2O, 20 °C)) were added to tubes, and tubes were stored in a dark for 5 minutes. Following that, tubes were transferred into cuvettes and absorbance was measured by a in spectrophotometer at 595 nm.

Table 1 Bradford Method Standard and Blank Preparation

	Blank	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
BSA (250 μg/mL) (μL)	-	4	8	16	32	48	64	80
Deionized Water (μL)	200	196	192	184	168	152	136	120
Commassie Brilliant Blue G250 (μL)	1000	1000	1000	1000	1000	1000	1000	1000

3.6 Pectin Degradation Test (Alcohol test)

Pectin degradation test is a qualitative analysis that is carried out to check for residual pectin after enzymatic treatment as described by Ervanuz (1985). Ethanol-HCl of 100 mL were prepared by adding 5 mL of 5% hydrochloric acid to 95 mL of 96% ethanol, then, 30 mL of enzyme treated 1% (w/v) pectin sample was mixed with 60 mL of prepared Ethanol-HCl solution. Samples that do not produce precipitation after 30 min were considered pectin free, reaching a complete depectinization.

3.7 Determination of Media and Physical Parameters for Pectinase Production

This stage consisted of change of nutrient sources and physical parameters to obtain better enzyme and specific activity. For this reason, various studies were reviewed and potential mediums with high activities were sorted out, then the potential media were kept modified to obtain best activity. During modification process, chemicals used in the study were replaced according to their functions and their concentrations were changed to attain and/or exceed current activity.

Consequently, components of growth/production medium were categorized under two categories, first category was chemicals used and their concentrations, second category was physical conditions of fermentation process.

First category was further divided into 4 sub categories, and these were carbon sources, nitrogen sources, activators, and salts. In this study, glucose and pectin (30 g/L in total) were used as carbon sources, whereas, ammonium sulfate, yeast extract and sodium nitrate (3.3 g/L in total) were used as nitrogen sources. Potassium chloride (0, 0.05 g/L) was used for homeostasis, inactivating protease and reducing/stopping production of aflatoxins. Lastly, predetermined amount of di-

potassium phosphate and magnesium sulfate penta-hydrate was used for potassium resource and for activating enzymes, homeostasis, cell division and suppressing stress, respectively.

Second category composed of three elements, which were pH (4.5, 5.5, 6.5), temperature of fermentation (30°C, 40°C, 50°C) and length of fermentation process (24h, 48h, 72h, 96h, 120h) (Table 2).

Table 2 Media and Conditions for SMF Pectinase Trials

	Pectin	Glucose	$(NH_4)_2SO_4$	K_2HPO_4	$MgSO_4.5H_2O$	KCl	Yeast Extract	$NaNO_3$	pH	Temp.	Time
M1	30 g/L	-	3.3 g/L	0.5 g/L	0.05 g/L	-	-	-	5.5	40 °C	72 hour
M2	20 g/L	10 g/L	3.3 g/L	0.5 g/L	0.05 g/L	-	-	-	5.5	40 °C	72 hour
M3	30 g/L	-	3.3 g/L	0.5 g/L	0.05 g/L	0.05 g/L	-	-	5.5	40 °C	72 hour
M4	30 g/L	-	1.65 g/L	0.5 g/L	0.05 g/L	0.05 g/L	1.65 g/L	-	5.5	40 °C	72 hour
M5	30 g/L	-	1.65 g/L	0.5 g/L	0.05 g/L	0.05 g/L	-	1.65 g/L	5.5	40 °C	72 hour
M6	30 g/L	-	1.1 g/L	0.5 g/L	0.05 g/L	0.05 g/L	1.1 g/L	1.1 g/L	5.5	40 °C	72 hour
M7	30 g/L	-	1.1 g/L	0.5 g/L	0.05 g/L	0.05 g/L	1.1 g/L	1.1 g/L	4.5	40 °C	72 hour
M8	30 g/L	-	1.1 g/L	0.5 g/L	0.05 g/L	0.05 g/L	1.1 g/L	1.1 g/L	6.5	40 °C	72 hour
M9	30 g/L	-	1.1 g/L	0.5 g/L	0.05 g/L	0.05 g/L	1.1 g/L	1.1 g/L	5.5	50 °C	72 hour
M10	30 g/L	-	1.1 g/L	0.5 g/L	0.05 g/L	0.05 g/L	1.1 g/L	1.1 g/L	5.5	30 °C	72 hour
M11	30 g/L	-	1.1 g/L	0.5 g/L	0.05 g/L	0.05 g/L	1.1 g/L	1.1 g/L	5.5	30 °C	24 hour
M12	30 g/L	-	1.1 g/L	0.5 g/L	0.05 g/L	0.05 g/L	1.1 g/L	1.1 g/L	5.5	30 °C	48 hour
M13	30 g/L	-	1.1 g/L	0.5 g/L	0.05 g/L	0.05 g/L	1.1 g/L	1.1 g/L	5.5	30 °C	96 hour
M14	30 g/L	-	1.1 g/L	0.5 g/L	0.05 g/L	0.05 g/L	1.1 g/L	1.1 g/L	5.5	30 °C	120 hour

3.8 Statistical Analysis

Statistical analyses were carried out using RStudio to test the significance of different variables (ammonium sulfate, pH, Temperature and Time) on pectinase activity by using regression analysis, analysis of variance (ANOVA) and multivariate analysis (Principal Component Analysis).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 *Aspergillus* species

Environmental samples (16) collected from various places (soils, tree roots, damp surfaces, etc.) were initially inoculated to selective agar (RBC) for 48 hours at 27° C to differentiate molds and yeasts. After initial inoculation, four suspected colonies of *Aspergillus* (black colonies with white mycelium) were transferred to fresh selective media to isolate pure colonies. Later on, colonies were transferred to PDB broths and kept for 48 hours at 27° C and left in incubator until white mycelium was formed. Spores were collected with the help of an inoculating loop and transferred into fresh PDB and glycerol mix and frozen for long storage at - 30° C (150µL and 850µL, respectively).

Species obtained were then tested for pectinase production by using medium described by El Enshasy (2018) (M1, Table 2). The strain with the highest activity (4.08 IU/ml) then chosen as main strain for further tests and sent to a private laboratory for determination of species and confirmed to be *Aspergillus brasiliensis*.

Aspergillus brasiliensis is one of the most common species of *Aspergillus* genus and the diseases caused by brasiliensis is quite rare compared to other Aspergillus species (aspergillosis). Yet, these species are the most common source (Rao et al., 2022) of otomycosis in humans. These species unable to produce aflatoxins (Health Canada, 2019), are mostly used in pharmaceutical and food industry for fermentation purposes.

4.2 Enzyme activity and Specific activity

Enzyme activity of the isolate was determined using DNS method with different media (Table 3). Highest activities obtained in M14 and M10, as 9.99 and 9.81 IU/mL, respectively. It should be noted that results from M11, M12, M10, M13, and M14 were obtained using same medium and samples were taken at 24 hour intervals (Figure 4).

For specific activity determination, Bradford (1976) method was used with highest enzymatic activity and it was determined that highest specific activity was obtained in sample M12, as 148.42 IU/mg.

When the samples were collected at 24 hour intervals, it was observed that enzyme activity slightly reduced after 72 hours and it reached the highest value at 120 hours. However, specific activity was reached highest level at 48 hours and started to decrease after 48 hours of fermentation. Possible reasons of slight decrease in specific activity can be contributed to temperature fluctuations, protease activity, protein denaturation as suggested by Otu and coworkers (2015).

The study involved data with a lack of replicates, precluding the ability to obtain multiple measurements for each condition. Nevertheless, it was aimed to derive information about the variability using the standard deviation based on the available data points. It should be noted that the standard deviation was computed in Rstudio from the limited available data points without replicates, serving as a descriptive statistic. However, it should be kept in mind that the obtained value might not fully capture the genuine variability within each condition due to the absence of multiple measurements. Consequently, it is emphasized that the standard deviation is

presented solely for informational purposes and should be subject to cautious interpretation.

Table 3 Enzyme Activity and Specific Activity of Pectinase Producing *Aspergillus brasiliensis*

	Time	Enzyme activity	Specific activity
M1	72 hour	4.08± 1.83 IU/ml	
M2	72 hour	5.05± 1.83 IU/ml	
M3	72 hour	6.53± 1.83 IU/ml	104.46 IU/mg
M4	72 hour	7.02± 1.83 IU/ml	
M5	72 hour	7.10± 1.83 IU/ml	
M6	72 hour	7.15± 1.83 IU/ml	
M7	72 hour	6.07± 1.83 IU/ml	
M8	72 hour	6.22± 1.83 IU/ml	
M9	72 hour	4.42± 1.83 IU/ml	
M10	72 hour	9.81± 1.83 IU/ml	137.50 IU/mg
M11	24 hour	6.67± 1.83 IU/ml	7.30 IU/mg
M12	48 hour	8.36± 1.83 IU/ml	148.42 IU/mg
M13	96 hour	9.01± 1.83 IU/ml	
M14	120 hour	9.99± 1.83 IU/ml	116.13 IU/mg

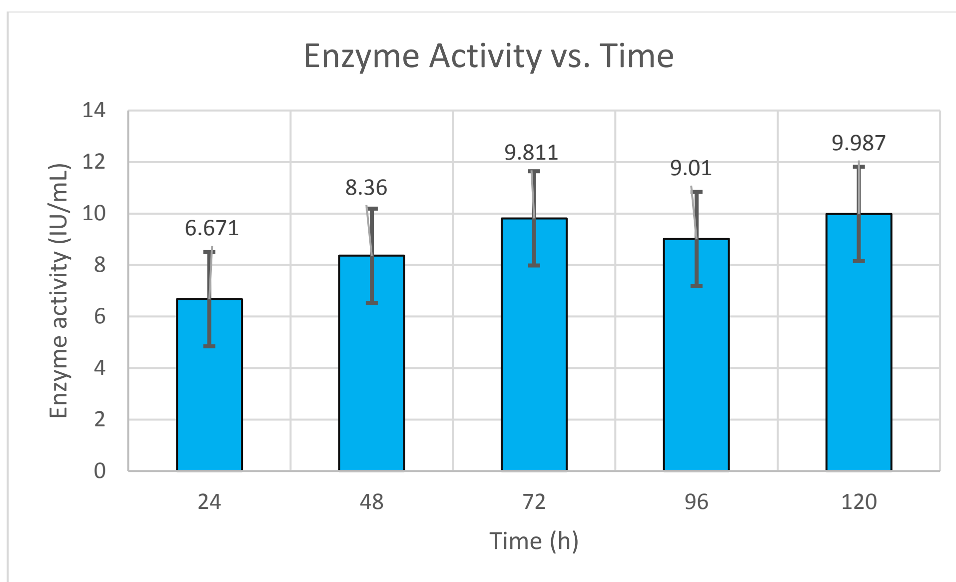

Figure 4. Enzyme activity versus Time

4.3 Pectin Degradation Test (Alcohol test)

Experiment was carried out as stated previously and results (Figure 5) showed that the obtained enzyme was capable of depectinization since both Sample A and Sample B is clear whereas, gel formation occurred in Sample C. This is due to reaction between methyl groups of pectin backbone and ethanol, and precipitate as gel (Sample A is commercial pectinase enzyme, Sample B is enzyme produced by *Aspergillus*, and Sample C is control).

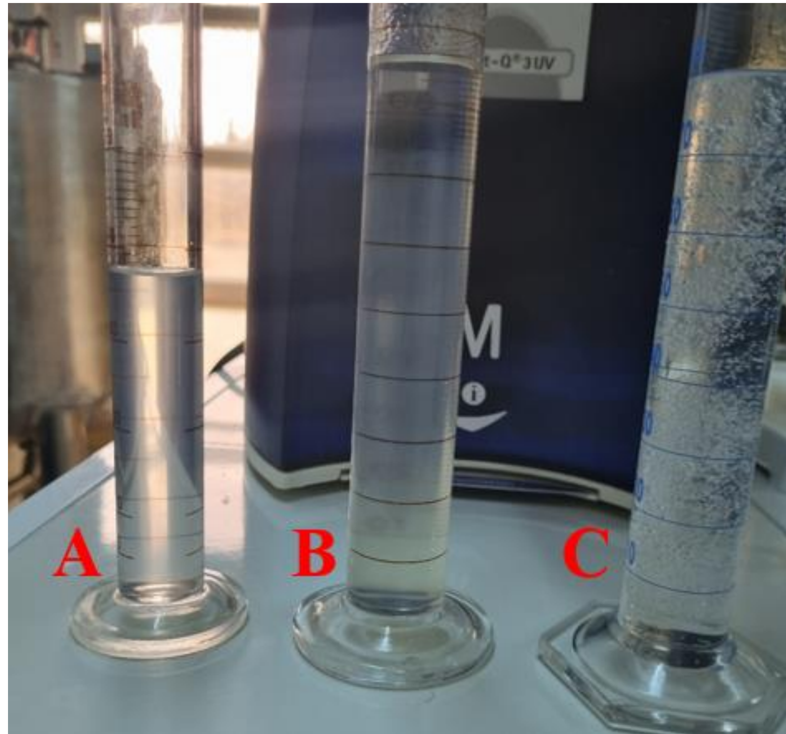

Figure 5. Sample depectinization experiment

4.4 Statistical Analysis

Statistical analysis was carried out in Rstudio in order to determine the effects of ammonium sulfate, pH, temperature and time on enzymatic activity. Analysis included regression analysis, analysis of variance (ANOVA) and multivariate analysis (Principal Component Analysis).

According to results obtained from regression analysis following model was obtained:

$$Enzyme\ activity = 6.1127 - 0.5769 * Ammonium\ sulfate + 0.0750 * pH - 2.1103 * Temperature + 0.7290 * Time$$

Equation 3

In this model, enzyme activity represents the response variable (enzyme activity). The terms ammonium sulfate (not significant, $p = 0.10823 > 0.05$), pH (not significant, $p = 0.91218 > 0.05$), temperature (significant, $p = 0.00115 < 0.05$), and time (significant, $p = 0.03584 < 0.05$) were the independent variables (factors) that have been included in the analysis. The coefficients (-0.5769, 0.0750, -2.1103, and 0.7290) indicated the estimated effect of each factor on the enzyme activity, controlling for the other factors. The multiple R-squared value was 0.8192, indicating that the model explained 81.92% of the variance in the response variable. This model explains the variance in response variable by including both significant and not significant components, because it can offer a more complete understanding of underlying relationships, account for potential confounders, and enhance the model's stability and generalization capabilities. The adjusted R-squared value was 0.7389, considering the number of predictors in the model. The residual standard error was 0.935, representing the average distance between the observed values and the predicted values. The overall F-statistic had a p-value of 0.002128, indicating that the model was statistically significant. Standard error for these experiment found to be 1.829836.

Analysis of variance (ANOVA) showed that the factors ammonium sulfate, temperature, and time were significant in explaining the variability in the response variable, and pH was not significant in this model.

According to multivariate analysis (Principal Component Analysis), the principal component analysis indicates that PC1 explained 33.44% of the variance, PC2 explains 25% of the variance, and the remaining components explain the rest of the variance.

CHAPTER 5

CONCLUSION & RECOMMENDATIONS

In this study, the aim was to isolate a wild *Aspergillus* species with potent pectinase production capabilities and the absence of aflatoxin production. Among the four isolates obtained, one demonstrated superior pectinase enzyme activity compared to the others, and this isolate was selected for further investigation.

Through initial screening for pectinase production, it was found that the chosen isolate exhibited high enzyme and specific activity when cultured at 30 °C for 72 hours, using multiple nitrogen sources, including ammonium sulfate, yeast extract, and sodium nitrate.

The results of this study hold promise for industrial implementation, particularly employing the submerged fermentation (SMF) method. The enzymes produced can be further purified using techniques such as dialysis and chromatography.

Aspergillus brasiliensis, the selected isolate, is an ideal candidate for application in the pharmaceutical and food industries due to its tolerance to high temperatures and pH levels, along with its inability to produce aflatoxins and ochratoxin A.

Further study is needed to optimize the enzyme activity and specific activity of the produced pectinase enzyme. Additionally, isolates obtained from extreme environments such as low water activity, high temperature, high acidity/alkalinity

should be tested for pectinase production to obtain wide variety of enzymes capable of working under various physical conditions. Furthermore, production of other enzymes such as amyloglucosidase, cellulase, β-glucanase and lipase should be tested.

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APPENDICES

A. Chemicals and Supplier Information

Table A. 1 Chemicals and Supplier Information

Chemicals	Suppliers
Pectin	ALFASOL
Glucose	Sigma-Aldrich
Ammonium Sulfate	BIOBASIC
Magnesium Sulfate Pentahydrate	MERCK
Dipotassium phosphate	MERCK
Potassium Chloride	ISOLAB
Yeast Extract	BIOBASIC
Sodium Nitrate	MERCK
Rose Bengal Chloramphenicol Agar	MERCK
Buffered Peptone Water	MERCK
Citric Acid	MERCK
Acetic Acid	MERCK
Hydrochloric Acid	MERCK
Sodium Hydroxide	MERCK
Bovine serum albumin	BIOBASIC
Ethanol	ISOLAB
Comassiae Brilliant Blue G250	Sangon Biotech (Shangai) Co. Ltd.
Immersion oil	ZAG Kimya
D-galacturonic acid	Sigma-Aldrich
3,5-dinitrosalicylic acid	Tokyo Chemical Industry
Sodium Acetate	ISOLAB
Glycerol	Sigma-Aldrich
Tween-20	Sigma-Aldrich

Potassium phosphate	MERCK

B. Buffers and Solutions

1. Acetate Buffer

Prepare 800 mL of deionized water, add 7.721 g of Sodium Acetate and 352.5 mg of Acetic Acid to the solution. Adjust solution pH to 4.5 using HCl and NaOH. Add deionized water until the volume is 1 L.

2. Citrate Buffer

Prepare 800 mL of deionized water, add 24.269 g of Sodium Citrate dihydrate and 3.358 g of Citric Acid to the solution. Adjust solution pH to 6.0 using HCl and NaOH. Add deionized water until the volume is 1 L.

3. Potato Dextrose Broth

Boil 200 g sliced, unpeeled potatoes in 1 liter deionized water for 30 min. Filter through cheesecloth and save effluent. Mix in 20 g dextrose with effluent and boil to dissolve. Autoclave 15 min at 121°C. Store in 4° C until use.

4. DNS Reagent

Dissolve 5 g of 3,5-dinitrosalicylic acid in 100 mL of deionized water at 80° C. Mix the solution with 100 mL of NaOH, 2 N and 150 g of potassium sodium tartarate. Complete the volume with deionized water to 500 mL.

C. Standard Curve for Enzyme Activity

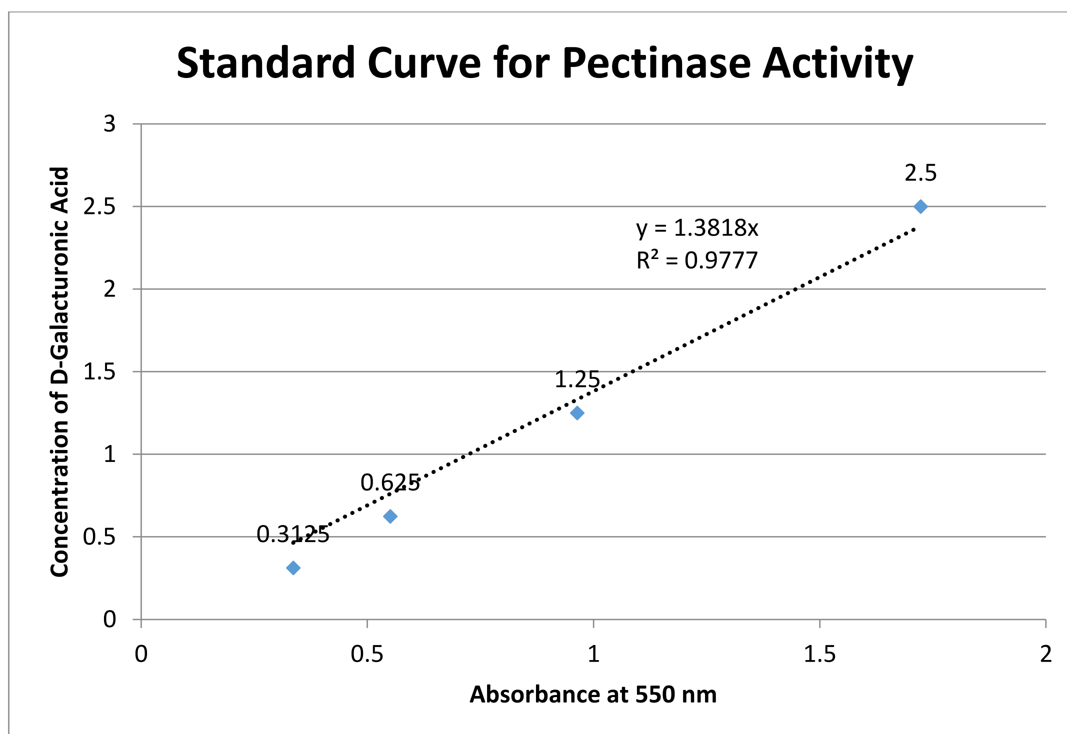

Figure C. 1 Standard Curve for Pectinase Activity

D. Standard Curve for Bradford Method

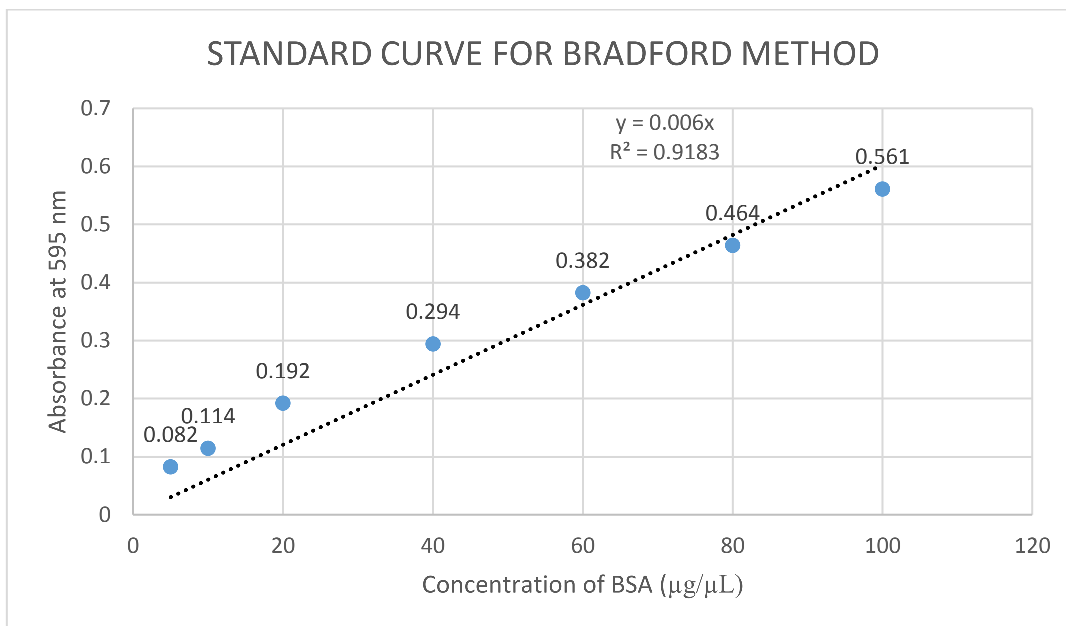

Figure D. 1 Standard Curve for Bradford Method