

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**DEVELOPMENT OF A NOVEL EXO-POLYGALACTURONASE
FOR AGRICULTURAL APPLICATIONS**

ERSİN KARATAŞ
A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

GEBZE
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MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
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SUMMARY

Polygalacturonases are a group of enzymes under pectinolytic enzymes related to enzymes that hydrolyze pectic substances. Polygalacturonases have been used in various industrial applications such as juice clarification, retting of plant fibers, wastewater treatment, drinks fermentation, and oil extraction.

The thesis was evaluated at the heterologous expression, purification, biochemical characterization, computational modeling, and performance in apple juice clarification of a new exo-polygalacturonase from *Sporothrix schenckii* 1099-18 in *Pichia pastoris*.

Two different pPIC9K plasmids were constructed with native signal sequence-ssexo-pg and alpha signal sequence-ssexo-pg separately. Protein expression and purification performed after transformation of *Pichia pastoris*. Biochemical and structural analyses were performed by using purified enzyme.

The optimum growth conditions for expression are induction with 1% methanol for 3 days at 30 °C. The purification of SsExo-PG was achieved by using Ni-NTA chromatography. The enzyme was found to have a molecular mass of approximately 52 kDa. The enzyme kept its activity about 50% at room temperature and +4 °C for 8 weeks. Structural analysis revealed that the catalytic residues of SsExo-PG are somewhat similar to other Exo-PGs. Cu^{2+} was found to enhance SsExo-PG activity while Ag^{2+} and Fe^{2+} almost completely inhibited enzyme activity. Kinetic properties of SsExo-PG were calculated against PGA and found 0.5868 μM and 179 s^{-1} for K_M and k_{cat} respectively.

The enzyme reduced turbidity up to 80% thus enhanced the clarification of apple juice. SsExo-PG showed promising performance when compared with other commercial pectinolytic enzyme mixtures.

Keywords: *Sporothrix schenckii* 1099-18, Exo-Polygalacturonase, Heterologous Expression, *Pichia pastoris*, Apple Juice Clarification,

ÖZET

Poligalakturonazlar pektin substratlarını parçalayan bir grup enzimlerdir. Yağ ekstraksiyonu, fermente içecek üretimi, meyve suyu durultması ve atık suların giderilmesinde poligalakturonazlar kullanılmaktadır.

Bu çalışmada *Sporothrix schenckii* 1099-18 mikroorganizmasından elde edilen yeni bir ekzo-poligalakturonaz enziminin heterolog ifadesi, saflaştırılması, biyokimyasal karakterizasyonu, hesapsal modellemesi ve elma suyunu durulma performansı değerlendirilmiştir.

Çalışmada biri alfa sinyal sekansına sahip olan diğeri ise SsExo-PG'e ait sinyal sekansına sahip olan iki farklı pPIC9K tasarlanmıştır. Uygun sinyal dizisi içeren plazmid *Pichia pastoris*'e transforme edildi ve protein üretimi ile protein saflaştırması gerçekleştirilmiştir. Biyokimyasal ve yapısal analizler saf SsExo-PG ile gerçekleştirilmiştir. SsExo-PG saflaştırılması Ni-NTA kromatografi sistemi kullanılarak yapılmıştır. Enzimin molekül ağırlığı yaklaşık 52 kDa olarak belirlenmiştir. SsExo-PG depo kararlılığı olarak 8 haftaya kadar oda sıcaklığı ve +4 °C'de enzimatik aktivitesinin %50'ye yakını korumuştur. Yapısal analizler SsExo-PG'nin diğer Exo-PG'lere yapı olarak benzediğini ortaya çıkarmıştır. Cu^{+2} SsExo-PG aktivitesini attırırken Ag^{+2} ve Fe^{+2} enzim aktivitesinin nerdeyse tamamını inhibe etmiştir. Saflaştırılan enzimin poligalakturonik asite karşı K_M ve k_{cat} değerleri sırasıyla 0.5868 μM ve 179 s^{-1} olarak belirlenmiştir.

SsExo-PG elma suyu berraklaştırma deneylerinde türbiditeyi %80 oranında azaltmıştır. Çalışmada kullanılan ticari pektinolitik enzim karışımları ile karşılaştırıldığında umut verici performans göstermiştir.

Anahtar Kelimeler: *Sporothrix schenckii* 1099-18, Exo-Polygalacturonase, Heterolog İfade, *Pichia pastoris*, Elma Suyu Durultma,

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and</u> <u>Acronyms</u>	<u>Explanations</u>
μg	: Microgram
μL	: Microliter
ADE1	: Aba Deregulated Gene Expression 1
AOX1	: Aldehyde Oxidase 1 Gene
ARG4	: Argininosuccinate Lyase 4 Gene
BCA	: Bicinchoninic Acid Assay
BLAST	: Basic Local Alignment Search Tool
BMGY	: Buffered Glycerol Complex Medium
BMMY	: Buffered Methanol Complex Medium
bp	: Base pair
BRENDA	: Braunschweig Enzyme Database
CIA	: Chloroform-Isoamyl Alcohol
CRISPR-Cas	: Clustered Regularly Interspaced Short Palindromic Repeats
Da	: Dalton
DMSO	: Dimethyl Sulfoxide
DNA	: Deoxyribonucleic acid
DTT	: Dithiothreitol
<i>EcoRI</i>	: <i>Escherichia coli</i> RY13 I
EDTA	: Ethylenediaminetetraacetic Acid
FLD1	: Formaldehyde Dehydrogenase Gene 1
GAPDH	: Glyceraldehyde-3-Phosphate Dehydrogenase
HCl	: Hydrochloric Acid
HEPES	: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
k_{cat}	: Turnover number
kDa	: Kilodalton
KEX2	: Killer Expression Defective Gene
K_m	: Michaelis-Menten constant
LB	: Luria-Bertani
LiCl	: Lithium Chloride

M	:	Molar
MALDI-TOF	:	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
Min	:	Minute
mL	:	Milliliter
MM	:	Minimal Methanol
mM	:	Millimolar
MS	:	Mass Spectrophotometer
NCBI	:	National Center for Biotechnology Information Database
ng	:	Nanogram
Ni-NTA	:	Nickel-Nitrilotriacetic Acid
nm	:	Nanometer
NTU	:	Nephelometric Turbidity Units
OD	:	Optical Density
PCR	:	Polymerase Chain Reaction
PE	:	Pectinesterase
PEG	:	Polyethylene Glycol
<i>PEX8</i>	:	Peroxisomal Biogenesis Factor
PG	:	Polygalacturonase
PGA	:	Polygalacturonic Acid
pH	:	Power of Hydrogen
PL	:	Pectin Lyase
PLs	:	Pectin Lyases
PME	:	Pectin Methylesterase
<i>PmeI</i>	:	<i>Pseudomonas mendocina</i> I
PMG	:	Polymethylgalacturonase
RNA	:	Ribonucleic Acid
RPM	:	Rounds Per Minute
<i>SalI</i>	:	<i>Streptomyces albus</i> I Restriction Enzymes
SDS-PAGE	:	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Sec	:	Second
SOB	:	Super Optimal Broth
SOC	:	Super Optimal Broth with Catabolites
<i>STE13</i>	:	DipeptidylAainopeptidase A 13 Gene

TAE	:	Tris-Acetate-EDTA
TLC	:	Thin Layer Chromatography
TSS	:	Transformation and Storage Solution
U	:	Units
UniProt	:	Universal Protein Resource
USA	:	United States of America
V	:	Volt
YNB	:	Yeast Nitrogen Base
YPD	:	Yeast Nitrogen Dextrose
YPDS	:	Yeast Nitrogen Dextrose Sorbitol
<i>YPT1</i>	:	GTP-Binding Protein YPT1

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1. INTRODUCTION

One of the important compounds of the plant is pectin which is formed repeats of galacturonic acids accompanying by α -1,4 glycosidic bonds. It is being used for decades as a renewable resource in various industries such as medicine, agricultural biotechnology, and other industries. The breakdown of the pectin is typically accomplished by a group of enzymes, pectinases including PME, PLs, and PGs [1].

Enzymes that can degrade the pectins as well as PGA, classified according to their substrate specialness and the site of the bonds they hydrolase [2]. PGs divide into two branches. Endo-PGs can hydrolase PGA randomly and cause to produce digalacturonic acids and trigalacturonic acids, while other branch Exo-PGs hydrolyze the PGA to produce monogalacturonic acids [3].

In the fruit juice industry, pectin degrading enzyme recipes have been used for decades to produce unclouded juice of fruits. Pectinolytic enzymes requisites to gain unclouded and steady drinks and superior concentrates, which offer acceptable juice yield together with good process economics [4]. In many specific fields of industrial biocatalysts, it is crucial to explain the properties and purification of enzymes. According to this several scientists have focused on the enzyme to increase purity, yield, and diversity from different sources [5].

Pectinolytic enzymes have several applications in industry, especially in the clarification of wine and fruit juice. Also, pectinolytic enzymes used in the extraction of vegetable fibre degumming, oil extraction from some plants, fermenting of drinks, degumming, and wastewater treatment, and in the farming industry to feed cattle and poultry. Most of the pectinases used in industry obtained from members of the *Trichocomaceae* family. The main pectinolytic enzyme in fruit juice processing is PG which also obtain from fungi [6]. Fungal PGs exhibit different properties that are required by the various biotechnological applications such as oil extraction from plants, juice clarification, and bleaching. *Aspergillus niger* is one of the most used filamentous fungi used for producing microbial pectinases. For decades, many microbial hosts have been thoroughly inspected for PG production, but the efficiency is still reported as big issue that limits low-cost PG applications [7].

P. pastoris is the most popular microorganism to produce recombinant protein. Alcohol oxidase genes (*aox1* and *aox2*) are strongly inducible promoters that can be

inducible with methanol and play important role in *P. pastoris* expression system. Performing eukaryotic post-translational modifications, having a high growth rate, and producing a good rate of protein are other remarkable properties of *P. pastoris* expression system.

There are several studies about obtaining Exo-PGs from various organisms which conferred to the literature [5, 6]. Nevertheless, Exo-PGs obtained from *Sporothrix schenckii* 1099-18 are not reported which contains biochemical and physiological specifications. Thus, in this thesis, we reported in detail the secretion of a new Exo-PG from *S. schenckii* 1099-18 by using *P. pastoris* expression system. Then, the purified SsExo-PG was widely characterized biochemically and computational. Apple juice clarification potential of the enzyme was investigated by comparing it with the commercial enzymes.

2. LITERATURE SURVEY

2.1. Biotechnology

Today, biotechnological studies facilitate human life and eliminate health problems, provide significant comfort and convenience of the human life extension issues. Modern biotechnology studies find application opportunities especially in the fields of agriculture, animal husbandry, and health. The definition of biotechnology has gone through many stages until it takes its present form.

Biotech; is derived from the words “biology” and “technology” and the first known definition was made in 1919 by Karl Ershy as “processes in which raw materials are converted into new products with the help of biological systems” [8]. The State Planning Organization, which previously carried out the development studies of Turkey, defined Classical Biotechnology as “technological applications that use biological systems and living organisms and their derivatives to transform or create products or processes for a specific use”. The same institution defines Modern Biotechnology as “all *in vitro* nucleic acid techniques that eliminate natural proliferation and recombination barriers such as direct injection of recombinant DNA into cells or organelles or cell fusion applied between different taxonomies and that are not used by classical breeding and selection methods”.

Today, biotechnology is defined as the use of biological systems in the production of goods and services with methods that do not adversely affect human and environmental health [9, 10].

Biotechnology has applications in many areas such as food, agriculture, animal husbandry, and health. Due to the development of technology and the use of biotechnology in different areas and even the increase in its applications in areas of use, technology is in continuous development.

Biotechnology studies were tried to be managed more effectively by using a different method in the classification of biotechnology (Table 2.1). The eight colors determined represented eight different activity classes, and this color and class distinction has been used in practice [11].

Table 2.1: Colors and classification of Biotechnology.

Color	Classification
White	Industrial Biotechnology
Gray	Environmental Biotechnology
Red	Medical Biotechnology
Blue	Marine Biotechnology
Purple	Patents, intellectual property rights
Yellow	Food biotechnology, nutritional science
Black	Bioterrorism, biological warfare, biological crimes
Green	Agricultural Biotechnology

2.1.1. Industrial Biotechnology

Industrial biotechnology is not easy to define. It describes many applications of biotechnology. Every type of industry has a biotechnology component. Even electrical energy production may be supplemented or replaced by industrial biotechnology processes. Some biotechnology industries use modern industrial processes to produce materials originally made by organisms. For example, an oily substance called glycerin that is found in many hand lotions was at one time collected from yeast. Now, it is made using chemical reactions that convert other molecules into glycerol. Other biotechnology industries use the organism as the technology to make various products. Many biotechnology industries focus on modifying organisms to improve the productivity of another industry. For example, the military uses genetically enhanced plants to assist with the breakdown of explosives polluting soil and water [9].

The first biotechnology experiments were carried out between 6000 BC and 1700 AD. In these years, people selectively bred animals and plants for agriculture for their beneficial character. By constantly producing organisms with similar characteristics, they ensured that the entire population had these characteristics. Besides, they did not allow animals and plants with undesirable traits to breed and caused the loss of these unwanted traits. Many of these characteristics have improved food production, as advances in production technology have improved industrial production. For example, strong, meaty cattle were bred with other strong, meaty

cattle to produce strong and healthy offspring. Weak cows were not permitted to breed, and thus fewer weak cattle were produced. Another early type of biotechnology was fermentation (a process in which microorganisms convert sugars to alcohol) to make seasonings and beverages such as wine and beer. People recognized long ago that bacteria and fungi produced alcohol and flavors when grown under certain conditions [10].

Most advances in industrial biotechnology were driven by discoveries in biology. A better understanding of biology provides scientists with ways to make new products. It also provides scientists with novel ways to use organisms to accomplish tasks that are usually done by chemical manufacturing or humans. Compared with traditional chemical manufacturing technology, organisms that can produce useful chemical substances more simply and purely have been developed. Also, organisms can use fewer people to operate processes, thereby producing large amounts of chemical substances.

The invention of the properties of DNA in 1953 paved the way for industrial biotechnology. It provided the basis for understanding how organisms function. The discovery of the gene function in the 1960s made possible the first genetic engineering research in 1973. Eventually, modern industrial practices incorporated modern genetics to create industrial biotechnology.

Today, industrial biotechnology is one of the fastest-growing industries in the world. Industrial biotechnology overlaps with agricultural, environmental, and pharmaceutical (medical) biotechnology.

As mentioned earlier, agricultural biotechnology involves the use and modification of organisms to produce food. Environmental biotechnology uses organisms to clean the environment.

Pharmaceutical biotechnology involves the discovery and production of animal and human drugs. Industrial biotechnology provides various goods and services together with other types of biotechnology. Many of the ingredients found in food today are made using industrial biotechnology. Some form of industrial biotechnology is used to make one or more components of almost all common objects we use every day [11].

2.1.2. Agricultural Biotechnology

Agricultural Biotechnology is defined as "the series of scientific techniques that include classical breeding practices to produce microorganisms, plants, animals for special purposes in agriculture, or to improve or modify their products". Nowadays, depending on the developments in molecular biology, it is applied as obtaining new "biotechnological products" that are Genetically Modified or improved with recombinant DNA and gene editing (CRISPR-Cas) technology. It is known that since the first planting of genetically modified products in the world in 1995, production has increased exponentially and reached a very large area of 189.8 million hectares [11].

For nearly 10,000 years, man has begun to improve wild plants and animals for desirable traits through selection and breeding. This breeding function is maintained by raising domesticated livestock and agricultural products that we commonly use today. In parallel with the increasing population in the twentieth century, these selected features have gained more importance as yield increase, resistance to diseases and pests, adaptation to different ecological conditions, short vegetation period, improved nutritional, and quality values. In 1850, Gregor Mendel's determination of hereditary traits and the birth of modern genetics paved the way for very important developments in plant and animal breeding [12].

2.2. Pectinolytic Enzymes

Pectic substances are structural and complex polysaccharides found in the plant cell wall and middle lamella with large molecular weights. Soft plant tissues with high moisture content and fast-growing contain high amounts of pectin. It is thought to have a control role in the transportation of water and plant fluids during the growth phase of the plants [12]. There is no certain molecular weight of pectinolytic macromolecules. It varies between 25-360 kDa. Enzymes of great industrial importance that break down pectic substances are called pectinolytic enzymes or pectinases [13, 14]. Pectinases are enzyme groups that break down the homogalacturonic acid chains of pectic substances through depolymerization and esterification reactions. Pectin provides strength and structure to plant cells [15].

Mechanisms of action are given in Figure 2.1 [16]. According to the cleavage site, pectin-degrading enzymes are classified into hydrolases, which are composed of lyase/trans-eliminase including pectin lyase, pectinesterase, and polygalacturonase.

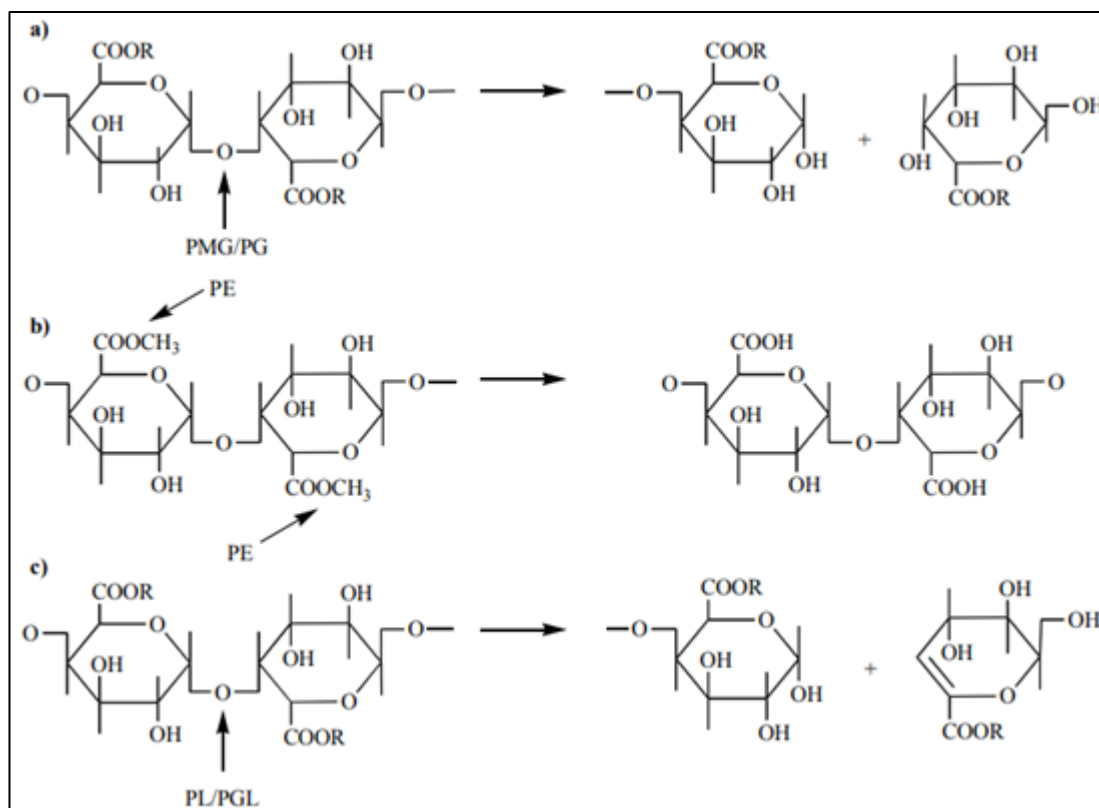


Figure 2. 1: Mechanisms of action for polygalacturonase (a) For PG R=H and PMG R=CH₃ (b) PE (c) For PGL R=H and PL R=CH₃. Arrows indicate where enzymes react with pectic substances. PMG; PG, (E.C. 3.2.1.15); PE, (E.C. 3.1.1.11); PL, (E.C. 4.2.2.10).

2.2.1. Polygalacturonases

PG is important pectin hydrolyzing glycanase, mainly involved in fruit softening during ripening [17]. It is believed to be the main cause of the dissolution of the intermediate flakes during maturation [18, 19] (Figure 2.2). Since the preferred substrate for these enzymes is D-galacturonic acid, researchers call it D-galacturonidase. [20]. Also, many researchers involved these enzymes have been reported in many fruits, such as peaches [21], pears [22, 23] and tomatoes [24]. When these enzymes hydrolyze the pectin material in the middle layer and cell wall, the activity of these enzymes will increase during maturation [25, 26].

Endo-polygalacturonases (Endo-PG; 3.2.1.15) are found in many microorganisms and plants such as fungi, bacteria, yeast. The best substrate for polygalacturonase is pectic acids. Pectic acids are depolymerized by hydrolysis by endo-polygalacturonase. They break the glycosidic bond close to the free carboxyl end in pectic acids. The rapid decrease in viscosity of the pectate solution is an indicator of PG activity. Even breaking a few bonds causes a 50% reduction in viscosity. PG activity decreases as the degree of esterification of the substrate decreases (Figure 2. 2).

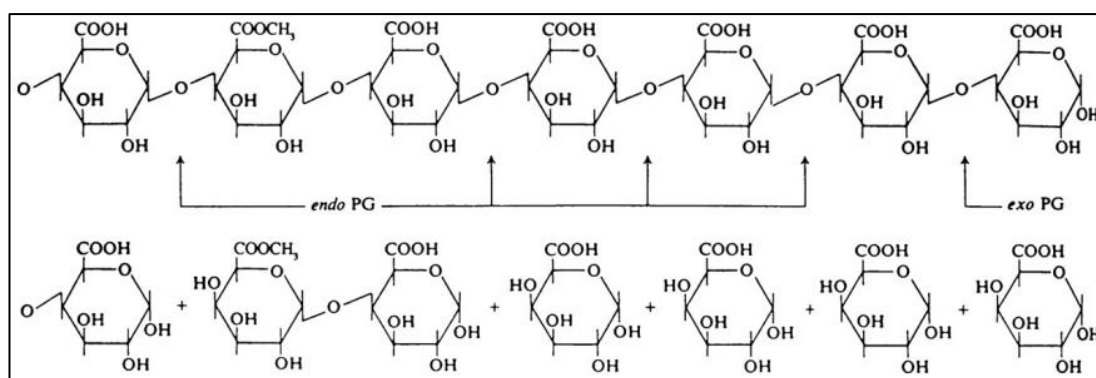


Figure 2.2: Exo-polygalacturonases and Endo-polygalacturonases effecting mechanism on the pectin.

Endo-polygalacturonases are produced by many plant pathogens or saprophytic fungi, bacteria, and some yeasts. A large number of endo-polygalacturonases have been purified, especially from fungi, in recent years. It has been determined that endo-polygalacturonases can have different molecular forms depending on microorganisms and various isoenzymes have been found. Most of these enzymes have a molecular weight of 35,000 Da and their glycoprotein origin has been observed in most of them [27].

Endo-polygalacturonases are specific for polygalacturonate. The calcium salt of the substrate is required for enzyme activity. Free tetragalacturonate anion is a competitive inhibitor [28]. Endo-polygalacturonase activity is determined by measuring the decrease in substrate density or the rate of increase of reducing groups. The rate and degree of hydrolysis of pectins rapidly decrease with the increase of esterification. Endo-polygalacturonases show different mechanisms of attack when oligogalacturonates are used as substrate. These differences are closely related to the structure of the active site of the enzyme, but especially to the state of

the catalytic groups and the size of the substrate-binding site. It is thought that carboxyl groups and some secondary alcohol groups in the substrate molecule play a role in the enzyme-substrate relationship [29].

Endo-pectinases are endo-enzymes that rapidly reduce the viscosity of the substrate solution by breaking the glycosidic bonds in the substrate. There is evidence that when the enzyme purified from endo-pectinase, *Aspergillus niger*, and *Aspergillus foncecanus*, which degrades tetra and tri-galacturonates with the lowest molecular weight, bound with its substrate, the carboxyl groups and tyrosine residues in the substrate are effective. As a result of the interaction of a pectic acid (substrate) solution with endo-polygalacturonases, its density decreases by 50%, indicating that only 2% of the α -1,4 glycoside bonds are hydrolyzed [30]. Exo-polygalacturonase hydrolyzes the α -1,4 glycoside bonds on the outer parts of the pectin polymer, revealing galacturonic acid residues. Exo-polygalacturonases are found in higher plants, some insects' intestinal systems, some fungi, and bacteria. The presence of this enzyme is usually made by the determination of free reduced groups of galacturonic acid monomers [31].

2.2.2. Industrial Applications of Pectinolytic Enzymes

Enzymes used in almost every field of the industry are generally obtained from microorganisms. Because microorganism-derived enzymes have advantages such as having very high catalytic activities compared to enzymes of plant or animal origin, not forming undesirable by-products, being more stable and cheaper and obtaining in large quantities. Enzymes obtained from microorganisms living high and low temperature, pH values or high concentration, conditions have started to be used extensively in the industrial field because they are resistant to extreme pH and temperature conditions. Due to the gradual development of enzyme technology, the diversity of usage areas of products, and their high economic value, various researches in the field of industrial enzymes of biotechnology gain more importance. The most used enzymes in the industrial field are protease, amylase, cellulase, xylanase, and lipase. Enzymes used in almost all areas of the industry are generally obtained from microorganisms. The reason for this is that microorganism-derived enzymes have higher catalytic activities than plant or animal-derived enzymes, do

not form unwanted by-products, are more stable and cheaper, and can be obtained in large quantities [32]. These microorganisms are selected not only for their ability to produce enzymes but also for the non-toxicity and pathogenicity of microorganisms. Since many enzymes used in industry today are of microbial origin, the use of microorganisms has increased in the use of industrial enzymes.

Pectinolytic enzymes have been used in food and juice production for more than 60 years. The main microorganism used in commercial pectinase production is *Aspergillus niger*. Pectinases are used in the preparation of fruit juices such as apple, pear, grape, citrus fruits, prunes, and vegetable juices such as tomato juice. With the use of pectic enzymes with high polygalacturonase activity, it has been achieved to stabilize the turbidity in fruit juices. Pectin degrading enzymes are used in the food industry, especially in clarifying fruit juices, facilitating filtration in fruit juice production and increasing yield, obtaining galacturonic acid, which is the starting material for vitamin C synthesis, in the wine industry, in the extraction of oils, in the preparation of pigments and cellulose fibers, in the fermentation of coffee and tea. They are used for new applications in production [33-36].

2.2.2.1. Agricultural Waste Saccharification

Wastewaters of citrus processing industries contain pectic substances that cannot be easily degraded by microorganisms during activated sludge treatment. However, these processes are known to have some weaknesses such as low efficiency, high cost, long processing time, and complexity of process processes due to the resistance of pectic substances to chemicals [37, 38]. It has been stated that the soft rot pathogen *Erwinia carotovora* secreting endo-pectin-lyase is beneficial in the first treatment of pectin wastewater. However, due to the dangerous nature of plant pathogens, the first indirect treatment with pectinolytic enzymes produced from bacteria was compared and it was determined that they decompose all pectic substances in wastewater [37]. So, the use of pectinase obtained from bacteria in selectively removing pectic substances from wastewater is an environmentally friendly, cost-effective alternative method. Alkalophilic soil isolate, *Bacillus* sp. Extracellular endo pectate lyase (optimally active at pH 10) produced from GIR 621 is effectively used to remove pectic substances from industrial wastewater [38].

2.2.2.2. Textile Processing

Pectinases are used in a step called enzymatic scrubbing (biological washing) in the textile industry. In this step, pectinase destroys the cotton cuticle structure by breaking down the pectin, thus removing the connection between the cuticle and the cotton fiber. Enzymatic scrubbing prevents the fabric from being affected by adverse environmental conditions. Besides, health problems are reduced by biological treatment instead of chemicals. The purpose of bleaching cotton is to decolorize natural pigments and give the fibers a pure white appearance. The most common industrial bleach is hydrogen peroxide. However, this application requires high amounts of chemicals and therefore much higher amounts of water for washing. Also, radical reactions of the bleaching agent with the fiber can cause serious damage due to a decrease in the polymerization degree. Due to all these problems, the enzymatic bleaching system has replaced the chemical bleach system. Thus, less fiber damage, better product, and savings are achieved in washing [1]. Enzyme-based detergent formulations are known as green chemicals. It finds a wide range of applications from laundry to the textile industry and dishwashing. The most commonly used detergent enzymes are hydrolases that consist of proteins, lipids, polysaccharides, and remove soils. The main classes are proteases, lipases, amylases, mannanases, cellulases, and pectinases [39].

2.2.2.3. Tea and Coffee Fermentation

Pectinases play an important role in the fermentation of tea and coffee. Pectinolytic microorganisms are used in the fermentation of coffee to remove the sticky layer in the coffee grains. Pectic enzymes are sometimes used to remove the fleshy layer on coffee grains. Reconstituted commercial enzyme preparations are sprayed into coffee beans at a dosage of 20 g per ton at 15-20 °C. The fermentation step of coffee processing was accelerated by enzymatic treatment and the time was reduced from 40-80 hours to 20 hours [37]. Fungal pectinases are also used in tea businesses. Treatment with pectinase speeds up the fermentation of the tea. However, enzyme dosage should be adjusted carefully to prevent damage to tea leaves [37]. It

also prevents the possibility of foaming of newly formed tea powders with the breakdown of pectins in tea [37, 38, 40].

2.2.2.4. Paper and Pulp Industry

Enzymes have begun to be used to solve some problems in paper and pulp production. Continuous filtration of water-suspended fibers and inorganic fillers (calcium carbonate and clay) is essential in papermaking. To absorb water from the leaves, fabrics that allow the passage of fibers and filling materials as well as water are used. In modern papermaking, restraints are added to the pulp to accelerate the absorption of water and protect the filler materials and fibers that make up the paper sheet. Various cationic polymers are widely used as retention agents. During the alkaline peroxide bleaching of the pulp, the polysaccharides can dissolve [39]. These dissolving polysaccharides are pectins or polygalacturonic acids. The complexity of polygalacturonic acids with cationic polymers (retention substances) depends on the degree of polymerization. While monomer, dimer, and trimer galacturonic acids do not form complexes with cationic polymers, hexamer, and longer chain polygalacturonates can form complexes with cationic polymers. Pectinases break down galacturonic acid polymers and prevent them from forming complexes with cationic polymers. Thus, fillers and fibers are preserved during the filtration of water, and better-quality paper is produced [37]. Enzymes have been used to solve some problems in paper and pulp production. Continuous filtration of water-suspended fibers and inorganic fillers (calcium carbonate and clay) is essential in papermaking. To absorb water from the leaves, fabrics that allow fibers and filling materials to pass through are used as well as water. In modern papermaking, restraints are added to the pulp to accelerate the absorption of water and protect the filler materials and fibers that make up the paper sheet. Various cationic polymers are widely used as retention agents. During the alkaline peroxide bleaching of the pulp, the polysaccharides can dissolve. These dissolving polysaccharides are pectins or polygalacturonic acids. The complexity of polygalacturonic acids with cationic polymers (retention substances) depends on the degree of polymerization. While monomer, dimer, and trimer galacturonic acids do not form complexes with cationic polymers, hexamer, and longer chain polygalacturonates can form complexes with cationic polymers.

Pectinases break down galacturonic acid polymers and prevent them from forming complexes with cationic polymers. Thus, during the filtration of the water, the fillers and fibers are protected, and better-quality paper is produced [37].

2.2.2.5. Animal Feed

In the early 1980s, various enzymes were added to poultry and other animal feeds. The first commercial success was the addition of glucanase to barley-based food diets. Enzymes were also tested in wheat-based diets. The xylanase enzyme is the most effective in this case. Generally, glucanase, protease, pectinase, and amylase enzymes are used in preparing enzymes and feed mixture. The purpose of adding enzymes to animal feeds is to obtain more meat yield by consuming the same amount of feed for animals. The addition of enzymes to feed increases the absorption of nutrients, reduces the number of feces, hydrolyzes the fibers to release the nutrients between them, and thus increases the nutritional value and enables animals to gain more weight by eating the same amount of feed. Petersen added an enzyme mixture containing pectinase, glucanase, and cellulase to the broilers' vegetable and protein mix foods. With the effect of these added enzymes, the change in the weight of the chickens was examined for 49 days and as a result, it was determined that the chickens gained significant weight [38].

2.2.2.6. Oil Extraction

Organic solvents used to be used to extract oils from oily plants such as canola, sunflower seeds, coconut, or olives. The most common of the organic solvents used are hexane, which is known to be carcinogenic. Today, fats are extracted using enzymes that break down the cell wall. Pectinases and other enzymes aid in the extraction process of oils by breaking down the cell walls of the oily plant. This method is preferred to organic solvents. Recently, enzyme preparations that break down the plant cell wall have been used in olive oil. Enzymes are added during the milling of olives. Thus, the oil is easily released by proper separation techniques. Olive oil yield has also increased as a result of enzyme treatment. Increase in yield; the pK depends on the temperature and dosage of the enzyme used. The enzyme

preparation Olivex, which has pectinolytic activity as well as hemicellulase and cellulolytic activity, shows a good oil extraction and better stability when stored. Besides, the polyphenol and vitamin E content, which preserves the oil's stability against rancidity, has also increased [37].

2.2.2.7. Fruit Juices Clarifying

Pectinases have potential biotechnological applications in fruit juice production industries [41]. It is accepted that pectic substances are the most important factors affecting the consistency, turbidity, and appearance of fruit juices [42]. By applying these enzymes to the fruit pulp, pectin is broken down, that is, the viscosity is reduced, and the fruit juice can be easily processed. These enzymes play an important role in softening, dissolving, and settling the pulp of fruits. The conventional method of precipitation of pectin containing fruit juice; includes centrifugation, enzymatic degradation of pectin, de-clouding with materials such as bentonite and gelatin, and filtration with diatomaceous earth. Pectinic substances in fruit juice can also be removed with membrane technology using ultrafiltration and microfiltration [43].

2.3. Recombinant DNA Technology

In several areas of biotechnology and industry recombinant DNA technology is being used for decades. The most common application of recombinant DNA is in basic research where technology is important for the most up-to-date studies in biological and biomedical sciences [44]. Recombinant DNA technology is used to identify, map, sequence, and determine their function. Recombinant DNA probes are used in the analysis of gene expression within individual cells and across tissues of whole organisms. Although recombinant proteins are widely used as reagents in laboratory experiments, they can also be used to examine protein synthesis in cells and organisms and to produce antibody probes [45].

2.3.1. Heterologous Gene Expression

The heterologous expression refers to the expression of a gene or part of a gene that does not naturally have the gene or gene fragment in the host organism. The gene is inserted into a heterologous host by recombinant DNA technology. After insertion into the host, the gene may be integrated into the host DNA, causing permanent expression, or non-integration, causing transient expression. The heterologous expression can be accomplished in many types of host organisms. The host organism can be bacteria, yeast, mammalian cells, or plant cells. This host is called the "Expression System". On the other hand, the homologous expression refers to the overexpression of a gene in its origin system.

Genes are usually expressed heterologously to study specific protein interactions. *Escherichia coli*, yeast (*Saccharomyces cerevisiae*, *Pichia pastoris*), immortalized mammalian cells and amphibian oocytes (i.e. unfertilized eggs) are usually used for research that requires heterologous expression. [46].

2.3.2. *Pichia* Expression System

Different high-scale production strategies have been developed to produce economically viable recombinant proteins using high-throughput expression systems [47]. Yeasts have become one of the promising host-vector systems in the production of recombinant genes compared to other eukaryotic and prokaryotic expression hosts [48]. For this purpose, yeasts such as *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica* are often preferred [47]. *P. pastoris*, a methylotrophic yeast, stands out as one of the most important yeast systems due to its advantages for heterologous protein production [49]. *P. pastoris* expression system used by Philips Petroleum Company in the 1970s for single cell protein production; It has become a convenient platform for the commercialization of a wide variety of protein products (such as enzymes, antigens, and antibodies) of human, animal, plant, fungal, and bacterial origin [50, 51]. Currently, there are more than 70 products produced in *P. pastoris*. Several pharmaceutical products manufactured in *P. pastoris* are also approved for human use by the US FDA [51]. In recent years, *P. pastoris* yeast named *Komagataella*

phaffii in the classification made according to 26S rRNA sequence was first produced by Guilliermond in 1919; It has been defined as yeast that is spherical or oval in shape, found in single or in pairs, and has a highly variable morphology [52, 53]. *P. pastoris* is a methylotrophic yeast and can provide all the carbon and energy required for its growth from methanol. When methanol is added to the growth medium of these yeasts, membrane organelles and peroxisomes containing enzymes that play a key role in methanol metabolism are strongly induced and increase massively [54]. *P. pastoris*; Its ability to reach high cell density in a short time, to eliminate endotoxin and bacteriophage contamination, to have high production efficiency, to easily manipulate well-characterized expression vectors, to have many promoters, and to be induced with different carbon sources, the expressed proteins; Polypeptide folding, ability to perform post-translational modifications such as glycosylation, methylation, acylation, low amount of secreted natural protein, and extracellular secretion of target proteins are among the reasons why it is preferred over other expression systems [55, 56]. Various genetic and physiological factors determine the efficiency of a recombinant system in *P. pastoris*. These factors are; codon optimization of the heterologous gene, gene copy number, the promoter used, the efficiency of transcription and translation processes, secretion signals, optimization of production conditions (temperature, pH, carbon source, production volume, feed profile, etc.), production method (intracellular, extracellular or cell surface) is the folding and secretion of protein [57]. There are many studies showings that with an increase in gene copy number, provided the level of secretion does not reach saturation, it can significantly increase productivity. This shows that there is a linear correlation between copy number and heterologous protein production [51]. Since episomal vectors (yeast plasmid) developed for *P. pastoris* are not stable, expression vectors are integrated into the genome of *P. pastoris*. Integrands created in this way can remain stable even in the absence of selective printing [58]. Expression vector in general; promoter sequence (AOX1, GAPDH, FLD1, PEX8, YPT1 etc.), single or multiple cloning site, transcription termination sequence-poly A tail, origin of replication, selection marker (ADE1, ARG4, G418, HIS4, URA3, Zeor, Ampr, etc.) and the secretion signal (PHO1, SUC2, pGKL, PHA-E, α -factor etc.) [55, 59]. The addition of foreign protein to expression vectors is usually performed in *E. coli*. Therefore, expression vectors are designed according to *E. coli* (vehicle

vector) / *P. pastoris* [55]. Many secretion signals are used in the *P. pastoris* expression system. However, the α -factor pre-pro secretion signal containing the pre region containing the 19 amino acid signal peptide from *S. cerevisiae* and the pro region consisting of 60 amino acids is the most widely used. In *P. pastoris*, the α -pre-pro sequence is mainly cut by the product of the *KEX2* gene (prohormone-endoprotease) and the product of the *STE13* (dipeptidyl aminopeptidase) gene [60].

2.4. *Sporothrix schenckii*

As a saprobe in the soil genus, *Sporothrix* are also parasitic organism found in animals and plants. This genus is anamorphic and shows similar properties to *Ascomycete* and *Optiostoma*. Also, their morphology and physiology are the same as fungi.

Although sporotrichosis has been reported from many parts of the world, it is mainly reported from hot and tropical countries (especially from Central and South America such as the USA, Mexico, Peru, Venezuela, Uruguay, Brazil, South Africa and Japan). The incidence and geographical distribution of sporotrichosis depend on the climate. The humidity of 90% and a temperature of 16-22 °C facilitate the breeding of *S. schenckii*. Therefore, its incidence is higher in autumn and early winter in tropical regions. Its incidence in a hyperendemic region in Peru has been reported to be 48-60 per 100,000 [61]. The number of cases reported in Turkey is limited. Only eight cases have been reported so far [62].

The agent is found in nature as saprophytes in soil and plants. *S. schenckii* was isolated from straw, splinter, bark, cotton, bush, lake thorn, and plowed soil. Because of their profession, vineyard and orchard workers, orchid and rose growers, florists, basket weavers, and carpenters are at risk. Human contamination is usually through the skin through products such as splinters, rose thistles, and straw (Figure 2.3) [63, 64]. Therefore, patients should be asked whether they are dealing with farming or gardening while taking their anamnesis. Another way of transmission is animal light and scratches. Although cats and armadillos are responsible for many cases, many reports of horses, dogs, spiders, mice, snakes, and birds have also been presented. The transition from an infected cheek of a mother to her child is the only human-to-

human transmission case reported up to now. Sometimes it is transmitted by inhalation of fungal conidia.

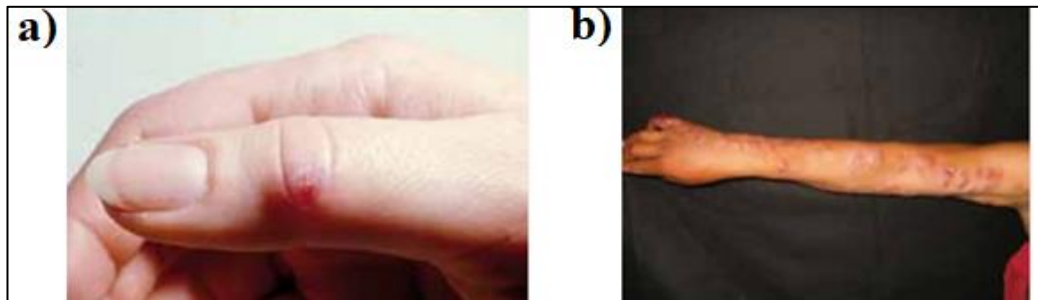


Figure 2.3: An ulcer nodule of fixed cutaneous sporotrichosis (a) is confined exclusively to the skin (b) with ‘ascending’ nodular (ulcerated or not) lesions developed along lymphatic vessels.

There is no reported industrial enzyme isolated from this organism. This thesis is the first study reported that an enzyme isolated from *S. schenckii* for agricultural application.

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Gene Constructs and Plasmids

The putative form (GenBank accession number: KJR88961.1) of SsExo-PG was analyzed (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) before the commercial synthesizes of the *ssexo-pg*. Conserved domains were determined using the Conserved Domain Database (<http://www.ncbi.nlm.nih.gov>), and the predicted signal sequences were identified using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>). The gene sequence was optimized for the codon usage preference of *P. pastoris* expression (<https://www.snapgene.com/>). The codon-optimized gene was submitted to GenBank (Accession number: MT628541.1) and the N-glycosylation sites, isoelectric point (pI), and molecular weight (Mw) of the SsExo-PG enzyme coded by the engineered *ssexo-pg* gene were estimated using the ExPASy Proteomics server (<http://www.expasy.org>).

The two designed *ssexo-pg* gene-containing plasmids with appropriate restriction cleavage sites tagged with a 6xHis affinity tag coding sequence at the end of the C terminus were synthesized by Genscript (USA) (Figure 3.1). The first design was a codon-optimized putative *ssexo-pg* gene with its own native signal peptide ligated into a plasmid pPIC9K called *ssexo-pg1* (Figure 3.2b).

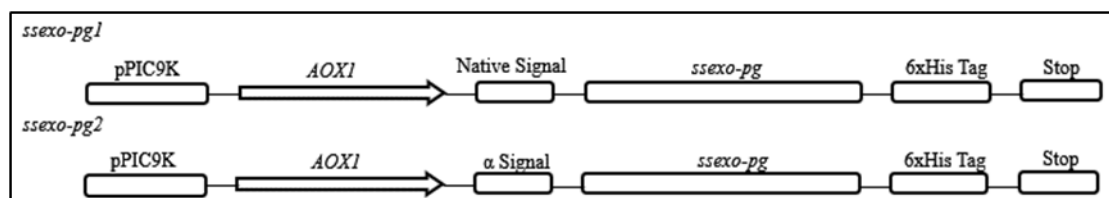


Figure 3.1: Plasmid constructs that designed for the study. *ssexopg-1* has the native signal *ssexo-pg* and 6xHis Tag whereas *ssexo-pg2* has alpha signal, *ssexo-pg* and 6xHis Tag.

The second designed plasmid had a codon-optimized putative *ssexo-pg* gene and included an alpha signal instead of using the predicted native signal as in the first design (Figure 3.2a).

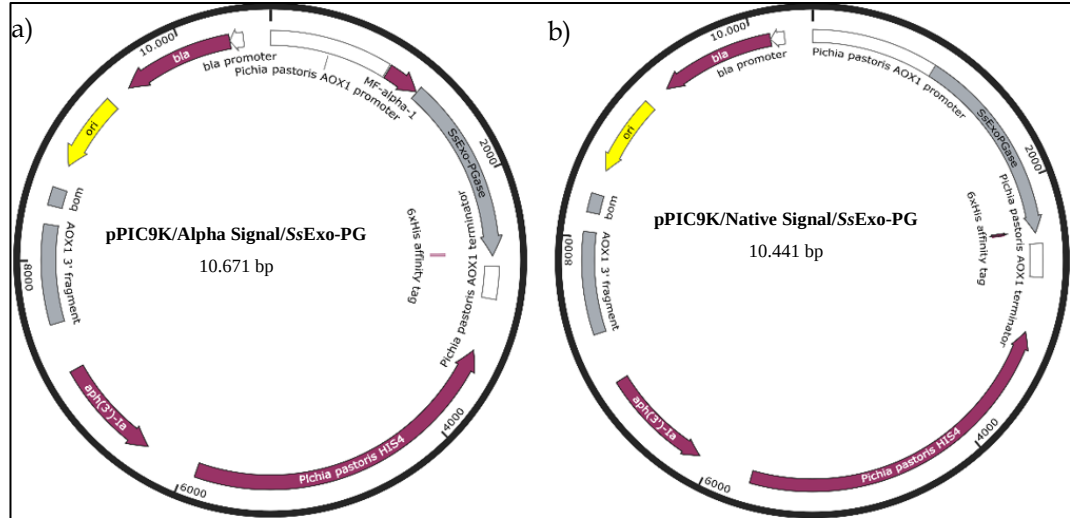


Figure 3.2: Designed vectors for expression of SsExo-PG. (a) Alpha signal but lack of signal sequence of SsExo-PG and vector (b) with a native signal but no alpha signal.

3.1.2. Microorganisms and Strains

The commercially synthesized (GenScript (USA)) SsExo-PG encoding codon-optimized genes were used for cloning, expression, and secretion. Sentromer DNA (Turkey) company made all sequencing experiments. *E. coli* and *P. pastoris* strains were purchased from Sigma-Aldrich (Germany). All the medium preparation steps were performed by following EasySelect *Pichia* Expression Kit manual (Invitrogen). *P. pastoris* expression vectors pPIC9K were obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA). The PGA used for examining Exo-PGs activity was purchased from Sigma-Aldrich (Schnelldorf, Germany), as was the DNA polymerase EndoH (Endoglycosidase H). A protein ladder (PI26620 Invitrogen, Carlsbad, CA, USA), all the kits used for experiments were obtained from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO, USA). Unless otherwise specified, the rest of the compounds used were bought from Sigma-Aldrich.

Escherichia coli TOP10 and *P. pastoris* GS115 (wild type, Mut⁺) cells were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA).

E. coli TOP 10 cells were used to amplify and purify recombinant plasmids. *P. pastoris* is a commonly used heterologous eukaryotic expression system. The advantages of the *Pichia* expression system are protein folding, post-translational modifications, the presence of processes in high eukaryotic cells such as protein processing, easy molecular manipulations, and inexpensive, rapid and high level of protein expression [65].

In this expression system, *P. pastoris*, a methylotrophic yeast, uses methanol as the sole carbon source. Methanol is oxidized to formaldehyde using molecular oxygen with alcohol oxidase enzyme encoded by alcohol oxidase (AOX1 and AOX2) genes. Besides, hydrogen peroxide, a toxic product, is formed in this reaction. To protect against this effect of hydrogen peroxide, *P. pastoris* performs the metabolism of methanol in an organelle called the peroxisome. To compensate for the weak affinity of alcohol oxidase to oxygen, it produces a large amount of this enzyme with the AOX, a powerful promoter. Thus, the product that is intended to be produced as heterologous is produced through this promoter [66].

3.1.3. Mediums and Buffers

Low Salt LB Medium (1 L):

- 10 g tryptone, 5 g yeast extract, 5 g NaCl are mixed in 950 mL deionized water. The pH is adjusted to 7 with 10 N NaOH and the final volume is completed to 1 L. The autoclave is done at 121 °C for 20 minutes and Geneticin to 25 µg/mL final concentration is added when it cools to ~55 °C. If Geneticin is added to the medium, it should be stored in the dark at 4 °C.

Low Salt LB Agar Plates (1 L):

- 15 g/L agar is added to the Low Salt LB medium before autoclaving. When the medium cools to ~55 °C after autoclave, Geneticin is added to 25 µg/mL final concentrations. Medium is poured into Petri plates. After the plates have hardened, they are inverted and stored at 4 °C. If Geneticin is added, Petri plates can be stored at 4 °C for 1-2 weeks.

YPD Medium (1 liter):

- 20 g peptone and 10 g yeast extract are mixed in 900 mL of deionized water. (If YPD agar plates are to be made, add 20 g of agar). The autoclave is done for 20 minutes. When medium cools to ~60 °C, 100 mL of 10X Dextrose is added. If desired, add 1 mL of 100 µg/mL of Geneticin into the medium. Liquid medium or agar plates added with Geneticin can be stored at 4 °C for 1-2 weeks.

YPDS Geneticin Agar (1 liter):

- 10 g yeast extract, 182.2 g sorbitol, 20 g peptone, 10 g agar are mixed in 900 mL of deionized water and autoclaved for 20 minutes. Autoclave for 20 minutes on the liquid cycle. When medium cools to ~60 °C, 100 mL of 10X Dextrose and 1 mL of 100 µg/mL Geneticin are added to the medium. Liquid medium or agar plates added with Geneticin can be stored at 4 °C for 1-2 weeks.

MM Agar Medium:

- 15 g of agar is mixed in 800 mL of deionized water and autoclaved for 20 minutes. When medium cools to 60 °C, add 100 mL of 10X YNB, 2 mL of 500X Biotin, 100 mL of 10X methanol, 1 mL of 100 µg/mL Geneticin. Medium is poured into Petri plates. After the plates have hardened, they are inverted and stored at 4 °C. If Geneticin is added, Petri plates can be stored at 4 °C for 1-2 weeks.

BMGY (1 L):

- 10 g of yeast extract and 20 g peptone are mixed in 700 mL of deionized water and autoclaved for 20 minutes. When medium cools to 60 °C, add 100 mL 1 M potassium phosphate buffer (pH 6.0), 100 mL 10X YNB, 2 mL 500X

Biotin, 100 ml 10X glycerol. For BMMY, add 100 ml 10X M instead of glycerol. The medium can be stored at 4 °C for 2 months.

Protein Purification Buffers:

Proteins purification from the supernatant was done by using HisTrap protein purification columns. Purification buffers are given below:

- Buffer 1: 1.38 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (20 mM), 14.61 g NaCl (500 mM), and 1.02 g imidazole (30 mM) were dissolved in 500 mL dH_2O and the pH was fixed to 7.4 using NaOH.
- Buffer 2: 1.38 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (20 mM), 14.61 g NaCl (500 mM), and 17.02 g imidazole (500 mM) were dissolved in 500 mL dH_2O and the pH was fixed to 7.4 using NaOH.
- Buffer 1 with 100 mM imidazole (50 mL): 42.50 mL Buffer 1- and 7.50-mL Buffer 2 were mixed.
- Buffer 1 with 200 mM imidazole (50 mL): 32 mL Buffer 1- and 18-mL Buffer 2 were mixed.
- Buffer 1 with 400 mM imidazole (50 mL): 10.50 mL Buffer 1- and 39.50-mL Buffer 2 were mixed.

Geneticin (Powder) solution:

- 100 mg/mL concentration of Geneticin is dissolved in HEPES (5 g/L, pH: 7.2) buffer and filtered through a 0.22 μm filter. It is stored at - 20 °C in the dark for 18 months.

Enzyme Activity Assay Buffers:

- Sodium acetate buffer (0.1 M, pH: 5.0): 5.772 g sodium acetate and 1.778 g acetic acid are mixed in 800 mL deionized water. 10 N HCl and 10 N NaOH are used to set the pH to 5. The final volume is completed to 1 L with deionized water.

- Britton Robinson buffer (0.1 M pH: 2.0-3.0 and 8.0-11.0): 0.1 M acetic, boric, and phosphoric acid mixture. The desired pH value is set at 10N HCl or 10N NaOH.

0.02% Biotin (500X):

- Filter sterilized solution which contains 40 mg of biotin dissolved in 200 mL of water can be stored at 2-8 °C. The solution can be stored for about 1 year.

YNB (10X):

- 134 g of YNB is dissolved in 1 L of deionized water and filtered through a 0.22 µm filters. It is stored at 4 °C.

20% Dextrose (10X):

- 200 g dextrose is dissolved in 1 L deionized water and autoclaved for 15 minutes. The solution can be stored for 1 year at room temperature.

5% Methanol (10X):

- 50 mL of methanol is mixed with 950 mL of deionized water. It is passed through 0.22 µm filters. It can be stored at 4 °C for 2 months.

10% Glycerol (10X):

- 100 mL of glycerol is mixed with 900 mL of deionized water and autoclaved for 15 minutes. The solution can be stored for 1 year at room temperature.

Potassium phosphate buffer, 100 mM pH 6.0:

- Potassium phosphate buffer (0.1 M, pH: 6.0): 2.405 g K₂HPO₄ and 11.73 g KH₂PO₄ are mixed in 800 mL deionized water. 10 N HCl and 10 N NaOH

are used to set the pH to 6. The final volume is completed to 1 L with deionized water.

SOB (1 L) and SOC (1 L) medium:

- 2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ were measured. Medium completed to 1 liter with ddH₂O and autoclaved. For SOC medium, glucose is added to the SOB medium with a final concentration of 20 mM and autoclaved.

TSS Buffer (pH: 6.5):

- 10% PEG (6000), 5% DMSO, 50 mM MgCl₂ is measured and autoclaved.

CIA, (50 ml):

- 48 mL chloroform, 2 mL Isoamylalcohol

Solutions Used for Agarose Gel Electrophoresis:

- 50X TAE Buffer (1 L): Measure 242 g Tris, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH: 8.0), and complete to 1 L with the final volume ddH₂O.
- 0.8% Agarose gel (40 ml): 0.32 g agarose, 3 µL Serva DNA Stain Clear-G, 40 mL 1X TAE.

Solutions Used for SDS-PAGE:

- 4X Sample Buffer (pH: 6.8): 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl.
- 10X SDS Running Buffer (1 L): 151.5 g of Tris, 720 g of glycine, and 50 g of SDS are dissolved in 1 L of ddH₂O.

- 30% Acrylamide-Bisacrylamide Solution (pH: 7.0): 290 g of acrylamide and 10 g of N, N'-Methylenebisacrylamide is dissolved in 1 L of ddH₂O and storage at 4 °C.
- Coomassie Staining Solution (2 Liter): 2 g Coomassie Blue (R-250) 900 mL methanol, 200 mL glacial acetic acid, and 900 mL ddH₂O.
- Destaining Solution: 400 mL methanol, 100 mL glacial acetic acid, and 500 mL ddH₂O.

Solutions Used for MALDI-TOF-MS: Matrix Solutions (20 mg/mL):

- Sinapinic acid: acetonitrile, 0.1% trifluoroacetic acid (70:30, v/v).
- α -Cyano-4-hydroxycinnamic acid (CHCA): acetonitrile and 5% formic acid (70:30, v/v)
- 2,5-dihydroxybenzoic acid (DHB): acetonitrile and 0.1% trifluoroacetic acid (TFA) (70:30, v/v)
- CHCA-DHB: Mix α -Cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid solution in 40:60 ratio (v/v)

3.1.4. Laboratory Equipment

Refrigerated centrifuge (Awel-CF48R and Nüve-NF400), ultracentrifuge (Beckman Coulter, Optima MAX-XP), plate centrifuge (Sigma), autoclave (Hirayama-HV110L), ultrapure water system (Gel-SpA), micropipettes (Axygen), dry air sterilizer oven (VWR-Dry-Line), (Biorad-SmartSpec 3000), nanodrop (Shimadzu-Biospecnano), sonicator (Diagenode-Biorupter Pico), shaking incubator (Inforce AG-CH4103 and Wisc, IS-20), precision weighing scale (Sartorius, BL-3100 and BL-210-S), magnetic stirrer (SCILOGX-MSPA), hotplate&magnetic stirrer (Daihan Scientific, MSH-20D), pH meter (HannaHI-2211), electroporator (BIO-RAD, Gene Pulser x-cell), vortex (Daihan Scientific, VM-10), water bath (Nüve, NB20), thermal cycler (Biometa, TRIO), incubator (Nüve, ES120), sterile cabinet (Nüve, MN090), plate reader spectrophotometer (Thermo, Varioskan), mini spin (Nüve, NF024).

3.2. Methods

3.2.1. Bioinformatic Analysis

Before the commercially *ssexo-pg* gene, its putative form (GenBank accession number: KJR88961.1) was analyzed [67]. Conserved domains were determined with the Conserved Domain Database [68]. Predicted signal sequences were identified using SignalP 4.1 [69] and MitoProt II-v1.101 [70]. The gene sequence was optimized for the codon usage preference of *P. pastoris* expression [71]. The codon-optimized gene was submitted to GenBank (Accession number: MT628541.1). N-glycosylation sites, pI (isoelectric point), and Mw (molecular weight) of the SsExo-PG enzyme coded by the engineered *ssexo-pg* gene were estimated ExPASy Proteomics server [72].

Two gene sequences for commercial synthesis were constructed based on the *ssexo-pg* gene sequence analysis. The first construct (*ssexo-pg*) was a codon-optimized *ssexo-pg* gene with its native signal peptide present that was cloned into pPIC9K. The other construct was a codon-optimized *ssexo-pg* gene with the alpha signal sequence.

3.2.2. Genetic Transformation

3.2.2.1. Bacterial Transformation

The method proposed by Chung et al. [73] was used to make cells competent and transformed. According to this procedure, the *E. coli* TOP10 cells to be competent were inoculated into 5 mL of liquid LB and incubated overnight. The next day, 100 μ L of pre-culture was added to 10 mL of fresh liquid LB. The growth amounts of the cells were measured with a spectrophotometer at 600 nm and expected to reach the desired OD value (0.4-0.5). When the culture reached the desired OD, it was centrifuged at 1500 $\times$ *g* for 5 minutes at 4 °C and the supernatant was discarded. After OD measurements, 10% of the remaining culture volume TSS was added and the cells were used for transformation after the pellet was thawed in ice.

10 μL of the ligation reaction was added to the Eppendorf tubes previously kept on ice and cooled. 50 μL of newly prepared competent *E. coli* TOP10 cells were added in Eppendorf tubes. After the tubes were mixed slowly, they were kept in ice for 30 minutes, and immediately afterward a 45-second heat shock was applied to the cells. It was then incubated in ice for 5 minutes. 950 μL SOC medium was added to each tube and incubated at 37 °C, 150 rpm for 80 minutes. Cells diluted after transformation were seeded into LB agar (25 $\mu\text{g/mL}$ Geneticin) medium plates to obtain single colonies. After overnight incubation at 37 °C, possible recombinant cells growing on the medium were collected and pure culture was prepared by inoculating the new LB agar medium plates. Plasmid isolation was performed from these cultures by the miniprep method [74]. The recombinant plasmids obtained were prepared for transforming into competent *P. pastoris* cells by cutting with *PmeI* restriction endonuclease.

3.2.2.2. Yeast Transformation

Recombinant plasmids were digested with *PmeI* restriction enzyme reaction to increase transformation efficiency (Table 3.1). After determining the amount of linear recombinant plasmids by Nanodrop, they were visualized in 1% agarose gel.

Table 3.1: Restriction endonuclease digestion reaction that prepared to linearize the recombinant plasmids.

Restriction Components	Final Concentration
Recombinant Plasmid	~6500-7000 ng
<i>PmeI</i>	40 U
Buffer B	1X
dH ₂ O	5 μL
Total	100 μL

At the end of the restriction reaction with *PmeI*, the restriction enzyme was treated for 20 minutes at 95 °C for inhibition of restriction enzyme inhibition. Recombinant plasmids digest and linear with *PmeI* restriction enzyme were first treated with phenol/chloroform isoamyl alcohol (CIA) for use in electroporation.

With this method, restriction enzymes are removed. Ethanol precipitation was used to concentrate linear recombinant plasmids. The Phenol chloroform extraction and ethanol precipitation method used is summarized below:

- For the 300 μL sample, 150 μL CIA and 150 μL phenol were added.
- Centrifuged for 5 min at $11.000\times g$. The supernatant was carefully collected with the pipette.
- Added 150 μL CIA and 150 μL phenol to the supernatant. Centrifuged for 5 min at $11.000\times g$. The supernatant was carefully collected with the pipette. Added 300 μL CIA to the supernatant. Centrifuged for 5 min at $11.000\times g$.
- The supernatant was carefully collected with the pipette. Added 1/10 volume of sodium acetate (3 M, pH 5.2). Added 2.5-3.0X volume (calculated after addition of sodium acetate) of at least 95% ethanol. Incubated at room temperature or on ice for at least 15 minutes. Centrifuged at $13.000\times g$ for 30 minutes at $-4\text{ }^{\circ}\text{C}$. Discarded supernatant and added 70% ethanol. Centrifuged again for 15 minutes and discarded supernatant. The pellet was dissolved in ddH₂O.

A modified protocol was used to generate competent yeast cells [75]. Single colony *P. pastoris* GS115 is inoculated as 2x1 cm on a YPD agar medium plate. It is incubated for 18 hours at $30\text{ }^{\circ}\text{C}$. Cells from the YPD agar medium plate are transferred to Eppendorf containing 1 mL of YPD broth. Cells are dissolved by making vortex. Centrifuge at room temperature for 3 minutes at $1500\times g$ and discard the supernatant. 1 mL sterile ddH₂O is added to the pellet and dissolved by pipette. The supernatant is discarded by centrifugation at $1500\times g$ for 3 minutes at room temperature. This step is repeated 3 times. Add 1 mL of the following solution (Table 3.2) to the pellet and incubate at 100 rpm for 20 minutes at $30\text{ }^{\circ}\text{C}$. The supernatant is discarded by centrifugation at $1500\times g$ for 3 minutes at room temperature.

Table 3.2: The culture medium prepared for the competent cell.

HEPES-NaOH (pH: 8.0)	40 μ L (1 M stock)
DTT	40 μ L (1 M stock)
LiCl	100 μ L (1 M stock)
Sorbitol	600 μ L (1 M stock)
ddH ₂ O	220 μ L

1 mL of cold 1M sorbitol is added to the pellet and the pellet is dissolved by pipette. The supernatant is discarded by centrifugation at 1500 $\times g$ for 3 minutes at 4 °C. This step is repeated for 750, 500, 250, 100 μ L sorbitol (1 M), respectively. At the last stage, 70 μ L of 1 M sorbitol is added to the pellet and dissolved by pipette. The competent cells obtained are used in electroporation.

Linearized 5 μ g plasmid mixed with 80 μ L competent *P. pastoris* GS115 was placed in a cold 0.2 mm electroporation cuvette and kept in ice for 5 minutes. Electroporation was performed at 1500 V, 25 μ F, 200 Ω . Cold 1 M sorbitol was added into the electroporation cuvette and taken into 15 mL falcon. Cells are incubated for 2 hours at 100 RPM at 30 °C. After incubation, cells were spread as 10, 25, 50, 100 and 200 μ L into YPDS medium (Geneticin 100 μ g/mL). The plates were incubated for 3-10 days at 28 °C until colonies were seen.

3.2.3. Sequence Analysis

The accuracy of gene structures in recombinant plasmids obtained as a result of plasmid isolation was demonstrated as a result of sequence analysis. All sequencing was done by Eurofins (Germany).

3.2.4. Polymerase Chain Reaction

The presence of transformed *ssexo-pg* gene constructs in *P. pastoris* GS115 cells was performed following the colony PCR protocol mentioned below (Table 3.3). Each colony taken with a sterile pipette tip was dissolved in 10 μ L of 0.02 M NaOH and kept in the microwave oven at the highest temperature for 3 minutes. Colonies were centrifuged at 3000 $\times g$ for 1 minute. 1 μ L was taken from the

supernatant and added as template DNA in the PCR reaction (Table 3.4). After colony PCR, samples were visualized on a 1% agarose gel.

Table 3.3: Primers used for colony PCR and reaction conditions.

MF-α Forward primer	5'-TACTATTGCCAGCATTGCTGC-3'	
AOX1 Forward primer	5'-GACTGGTTCCAATTGACAAGC-3'	
AOX1 Reverse primer	5'-GCAAATGGCATTCTGACATCC-3'	
ssexo-pg Reverse primer	5'-GACACCAATTCTTCTAACAGCAGG-3'	
Cycle	Temperature (°C)	Time (min)
1	95	1
35	94	1
	58	1
	72	1:30
1	72	10

3.2.5. Protein Purification

The transformed *P. pastoris* GS115 cells were grown in BMMY medium. Optimized conditions determined by previous experiments, were used for cell growth. Obtained culture were centrifuged at 11000xg, 4 °C for 30 min. than filtered using 0.45 μ m pore size filters before ultracentrifugation (MM Amicon Ultracel-30 K) to concentrate the SsExo-PG enzyme. The concentrated sample was loaded to a HisTrap FF 5 mL Ni²⁺-NTA column in an AKTA purifier FPLC system (GE Healthcare Biosciences). The collected fractions were analyzed using SDS-PAGE to determine the expression and purity level. The purified and re-concentrated SsExo-PG was stored at 4 °C for further analysis. The concentration of the purified SsExo-PG was identified using the BCA Protein Assay Kit. The expression and purification efficiency were monitored by %12 SDS-PAGE. Samples were prepared by mixing 15 μ L sample and 5 μ L 4X sample loading buffer. Samples were heated at 95 °C for 10 minutes then centrifuged at 14.000×g for 10 minutes. The SDS gel was placed in the tank then the tank was filled with running buffer. 5 μ L protein standard was loaded into the first well and 5 μ L of sample is loaded into the next wells. Then the

running was started with 80 V for 10 minutes and it was continued at 100 V for 1 hour.

3.2.6. Biochemical Analysis

3.2.6.1. Exo-Polygalacturonase Activity Assay

The whole-study DNS method [76] with PGA as a substrate was used to examine the activity of the purified SsExo-PG with a constructed standard curve. The standard curve for enzyme assay was prepared using five different concentrations of galacturonic acid (0.1, 0.2, 0.5, 1, 2, 4 mg/mL) (Figure A1.1) in distilled water. Three mL of each D-galacturonic acid sample was mixed with 3 ml of DNS solution (Dinitrosalicylic acid: 10 g, Phenol: 2 g Sodium sulfite: 0.5 g Sodium hydroxide: 10 g dissolved in 1 L distilled water). Each tubes were mixed with vortex and put into 90 °C water bath for 15 min. After developing the red-brown colour, test tubes were taken out from the water bath and 1 mL of 40% Rochelle salt was added to stop the reaction. The tubes were allowed to cool and finally, absorbance was measured at 575 nm.

3.2.6.2. Effect of pH, Temperature, Metal Ions and Solvents on Activity

Different buffers were tested to specify the optimal pH values of the purified SsExo-PG enzyme activity at 24 °C. We further investigated the effects of 100 mM sodium acetate buffer with a pH range of 4.0-6.0 and 100 mM Britton Robinson buffer with a pH range of 2.0-3.0 and 7.0-11.0 on SsExo-PG activity.

The specific activity of the enzyme was followed through temperatures ranging from 30 to 90 °C (in 5 °C increments) in optimum pH to determine optimum temperature.

Metal ions and organic solvents effect on enzymatic activity was determined. For this purpose, the metal ions of Ag^{2+} , Ca^{2+} , Co^{2+} , Fe^{2+} , Hg^{2+} , Li^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} (in measurements of 1, 10, and 100 mM) in sulfated forms (except $\text{Ca}(\text{NO}_3)_2$ due to low solubility) the organic solvents of dimethyl sulfoxide, isopropanol, methanol, ethanol, acetone, acetonitrile, cyclohexane, and ethyl acetate at different concentrations (10, 25, and 50%) (v/v).

3.2.6.3. Storage Stability

The evaluation of the storage stability of the various exo-polygalacturonase preparation for four different temperatures (20, 4, -20, and -80 °C) were used to determine the storage stability of purified SsExo-PG enzyme. The measurement of enzyme activity was carried out once a week for 8 weeks. The enzymatic assay was performed with a defined optimum pH, 100 mM sodium acetate buffer, and PGA substrate.

3.2.6.4. Kinetic Assay

The kinetic parameters (K_m , k_{cat} , k_{cat}/K_m) of SsExo-PG were calculated for different PGA concentrations [76]. Measurements are taken at 545 nm through a spectrophotometer after samples are cooled to room temperature. The formed curve was adapted to the Michaelis-Menten equation with GraphPad Prism 8.0 (GraphPad Software, San Diego, USA) by nonlinear least-squares regression: one unit was described as the amount of enzyme that transforms 1 μ mol of substrate per minute to the product. All experiments were carried out in triplicates.

3.2.7. Structural Analysis

3.2.7.1. Deglycosylation and MALDI-TOF MS Analysis

The purified recombinant SsExo-PG was deglycosylated by treatment with EndoH, and experiments were carried out according to manufacturer instruction (P0702S NEB, MA, USA). Deglycosylation and molecular weight were monitored using SDS-PAGE and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (Bruker Daltonics, Billerica, MA). MS was calibrated using appropriate proteins obtained from the manufacturer [77].

3.2.7.2. Homology Modeling and Molecular Docking Studies

The 3D homology model of SsExo-PG was generated using I-TASSER [78] and the published structure of *Aspergillus tubingensis* endo-xylogalacturonan hydrolase (XghA, PDB ID: 4C2L) [79] as a template. PGA (PubChem CID: 439239) substrate was docked into a SsExo-PG active site pocket *in silico* via the AutoDock platform [80]. Drawings were done using PyMOL (Molecular Graphics System, Version 2.0 Schrodinger, LLC). Amino acid sequences of the reviewed Exo-PGs and SsExo-PG from UniProt were aligned with Clustal Omega, and a figure was created with Geneious software (UniProt accession numbers; *Aspergillus niger*: Q27UB3, *Aspergillus tubingensis*: Q00293, *Neosartorya fumigata*: Q4WCZ8, and *S. schenckii* 1099-18: A0A0F2MLB4). The protein secondary structure was predicted using EMBOSS tool Garnier, and a figure was created using Geneious software [81].

3.2.7.3. Thin Layer Chromatography Analysis

The corresponding hydrolyzate was spotted on 8x10 cm silica gel 70G TLC plates after being concentrated 10 times. Mono-galacturonic acid, di-galacturonic acid, tri-galacturonic acid, and PGA were used as standards. The chromatography was performed using the ascending method; the mobile phase consisted of a 5:3:2 mixture of n-butanol: H₂O: acetic acid. For visualization of the spots, the dried plate was sprayed with 10% sulfuric acid in methanol followed by heating at 105 °C for 5 min.

3.2.8. Apple Juice Clarification of SsExo-PG

Three different commercial enzymes were used for turbidity experiments. Several different combinations of Enerzym HT (Glucoamylase, ERBSLÖH, Germany), Fructozym® P6-L (Arabanase, ERBSLÖH, Germany), and Fructozym® FLUX (Pectolytic enzyme, ERBSLÖH, Germany) with/without SsExo-PG were performed as follows: (1) Enerzym HT (36 mg/L) + Fruktozym P6L (24 mg/L) + Fruktozym Flux (19 mg/L), (2) Enerzym HT (36 mg/L) + Fruktozym P6L (24 mg/L), (3) Enerzym HT (36 mg/L) + Fruktozym Flux (19 mg/L), (4) Enerzym HT (36

mg/L) + SsExo-PG (15 mg/L), (5) Enerzym HT (36 mg/L) + SsExo-PG (30 mg/L), (6) Enerzym HT (36 mg/L) + SsExo-PG (45 mg/L), (7) SsExo-PG (15 mg/L), (8) SsExo-PG (30 mg/L), and (9) SsExo-PG (45 mg/L). The turbidity of the juice was measured using an HD 25.2 turbidimeter (Delta Hom, Padua, Italy) and expressed in nephelometric turbidity units (NTU) [82]. After each measurement, samples of the apple juice were heated to 60 °C and incubated for 1 h. Finally, incubation turbidity was analyzed using turbidity (% T660 nm) and color (A420 nm) measured with a UV-2700i UV-VIS (Shimadzu) spectrophotometer; these were then compared with the control.

3.2.9. Statistical Analysis

The statistical significance of the data obtained from the apple juice clarification treatments was evaluated by analysis of variance (ANOVA) performed using IBM SPSS (<https://www.ibm.com/tr-tr/products/spss-statistics/details>). The differences were considered significant with $p \leq 0.05$.

4. RESULTS

4. 1. Bioinformatic Analysis of SsExo-PG

The SsExo-PG sequence (Figure 4.1) showed the similarity between Exo-PGs from *Aspergillus niger* (60.2%), *Aspergillus tubingensis* (60.2%), *Neosartorya fumigata* (strain ATCC MYA-4609 / Af293 / CBS 101355 / FGSC A1100) (58.6%), *Neosartorya fumigata* (strain CEA10 / CBS 144.89 / FGSC A1163) (58.6%), and *Emericella nidulans* (58.5%) (Figure 4.2).

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ATGAGATTTCCTTCAATTTTACTGCAGTTTTATTGCGAGCATCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCA
CAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTGCTGTTTGCCATTTTCCAACAGCACAAATAACGGG
TTATTGTTTATAAATACTACTATTGCGAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTTACGTAGAATT
CGCTCCATCTAACGGTAATGGTAACGGAAACGGTAACGACTCCGCTCCATGTAAGCCAGTTTCTACTAGACCAACCGTTGTCGCTCATCCAA
AGCAACCTAGACTGCCAATTCCAATTCCACCACCAAGAAACAAGGTCTGCTGTGTTAAGACTCACGGTGACGGTGTACTGACGACTCCACT
TACATTATGTCCGCTTCCACGCTTGTAAACCACGGTGTCACGTTGTTTTAGAGCTGACACCACCTACTTGGTCGGTACTGCTATGGATTGGA
CCTTCTTGAAGGACATCGACATTTGGTGGTTCCGGTAGAACCATCACCGTTAACACTGTTGCTAACATCCCACCAGACATCCAGGGTACTATCC
AGTTCATAACGACACTGACTACTGGCAGGCTAACTCCTCAGATTCCGGTTTCCAGAACGTCACCTCATTCTCAAGCTTGGAGGTGAGAACG
TGTTGTTTACGGTGGTGGTACATTGCACGGTAACGGTCAAGTTTGGTACGACTTGCACGCTCAAGACACTGACACTTTGAGGCCAGTTTGG
TTGGTATTGACGGTCTGCAGAACTCCGTTGTTGCTGACTTGCACCTGAGACTGTCCCAATGTACTACCATTTGCTTGTAAACGCTCCAACGT
CGTTTCTCCGACATTGACATTTCCATCTCCAACCTCCAACGGTGCTGCTTCTTCTGCTCATCATGCTGCTAAGAACACTGACGGTTGGGAC
ACTTACAGATCTCCGATATCGCTATCTTGAACCTCCGTCGCTGTTAATGGTGACGACTGTGTTTCCCTTGAAGCCAACTCCACTCACATCTTGG
TTGCTTCCCTGCTCTTGTACTGGTTCACGGTATTTCCATTGGTTCCTTGGGTCATACGCTGGTGCTTACGACATCGTTGAACACGTTTGGGT
TGGTAACGTTTCTCTGCACGGTCTACTGATGGTGCCAGAAATCAAGTTTGGCCTAACACTCCATCTGCTTTTCCGGTGATCTTCAAGGTGG
TGTTGGCGGTGGTAGAGTTAGAAACATTACTACGACGGTATGTACATCGACGGTGTGCGACTACGCTATTGAGATCACTCAATGCTACGGTC
AGAAGAAGTTGACTGTTTGTGACGACGCCATCTTCTGACTTTGGCCGATGTCGTGTTTCAGAACTTCTACGGTGTACCTCCACTAAGT
ACGCTCCACAAATTTGGCTTGTTCCTCCTGCTACTTGTAGAGCTATTTCCGCTACCAACATCGACGTCGTTTCACTAGAGGTGG
TAACCAGGCCTACTGTTGAACCAGAACGCTACTGCCTTGGACTTGACTGTACTGACGTTTGAAGGGTTCAACTGA
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Figure 4.1: Nucleotide sequence of the codon-optimized *ssexo-pg* gene (1833 bp).

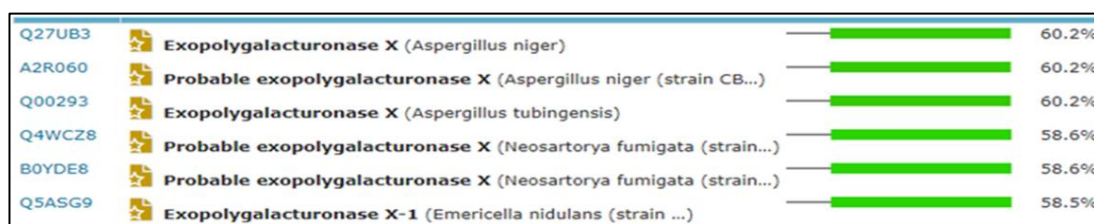


Figure 4.2: Amino acid sequence alignment of SsExo-PG with other well-known and published Exo-PGs. These Exo-PGs has been reviewed and proved that they have enzymatic activity.

The putative *ssexo-pg* was analyzed and found as 1434 bp, which encodes 478 amino acids. 1-18 amino acid sequence pattern *ssexo-pg1* and *ssexo-pg2* having

native signal peptide (1-18 aa) and α -mating factor (89 aa), respectively, for use in determining the effect of the signal peptides on SsExo-PG activity (Figure 4.3).

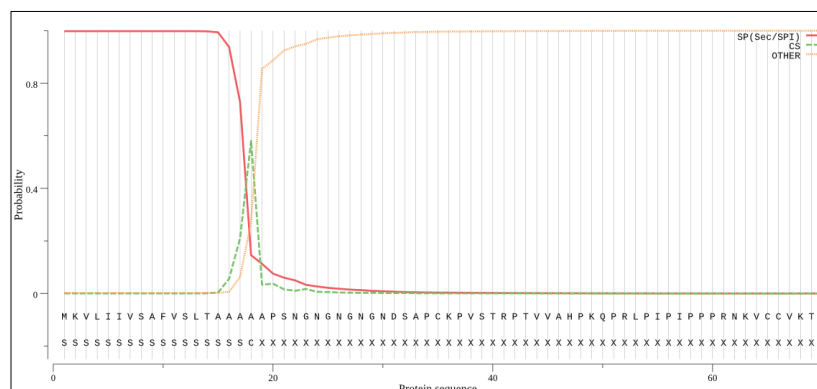


Figure 4.3: Signal sequence analysis (SignalP-5.0) of the *ssexo-pg* gene.

As expected, the Mw and pI values of the enzyme were calculated as 50.68 kDa and 6.02 respectively.

After determining the conserved domains, former publications about pectinases have been examined and analyzed the sequences of the related gene in the publications. Thus, the chosen gene for this thesis could be determined well. According to Chen et al. 2017, *RsPG2* gene from *Rhizoctonia solani* has been functionally analyzed it is indicated that NAD, DD, SHG, RIK motifs (Figure 4.4) are belonged to family 28 of the pectin degrading glycoside hydrolases [83].

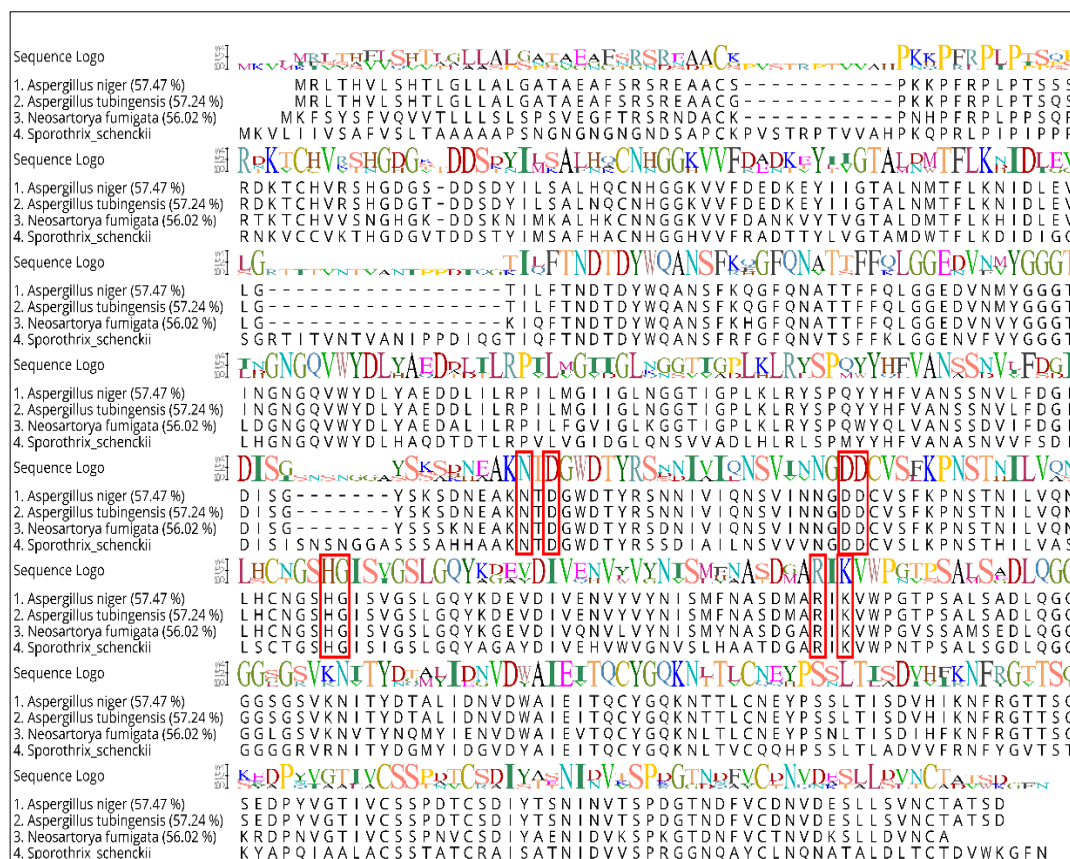


Figure 4.4: Multiple amino acid sequence alignments of Exo-PGs. The red rectangles indicate the strictly conserved residues among the PGs (UniProt accession numbers; *Aspergillus niger*: Q27UB3, *Aspergillus tubingensis*: Q00293, *Neosartorya fumigata*: Q4WCZ8, and *S. schenckii* 1099-18: A0A0F2MLB4).

4.2. Transformation of Plasmid into *E. coli*

Recombinant plasmids obtained after the ligation reaction were transformed into *E. coli* TOP 10 cells by heat shock. Recombinant cells were visualized on the plate with LB agar medium containing 25 µg/mL Geneticin. *E. coli* TOP 10 without recombinant plasmids was used as the control group (Figure 4.5). Cells growing on the plate were used as a recombinant plasmid source containing *ssexo-pg* gene constructs for later studies.

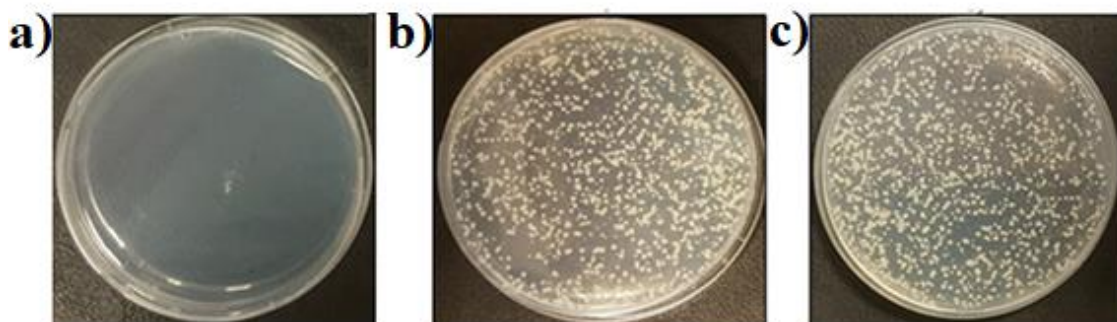


Figure 4.5: *E. coli* TOP10 colonies developing on LB agar medium as a result of transformation. (a) Control (b) *ssexo-pg1* (c) *ssexo-pg2*.

4.2.1. Agarose Gel Electrophoresis Analysis

The presence of recombinant plasmids in *E. coli* TOP10 host cells was performed by *Eco*RI and *Sal*I restriction endonuclease double digestion enzyme reactions and visualized in 1% agarose gel (Figure 4.6). In-gel images, the *ssexo-pg1* and *ssexo-pg2* gene constructs were also determined by the pPIC9K plasmids they were ligated.

Thus, the accuracy of plasmid isolated recombinant plasmids and *ssexo-pg* gene constructs have been proven. These plasmids were re-isolated and prepared for sequence analysis to determine that the *ssexo-pg* gene constructs are properly ligated into the recombinant plasmids.

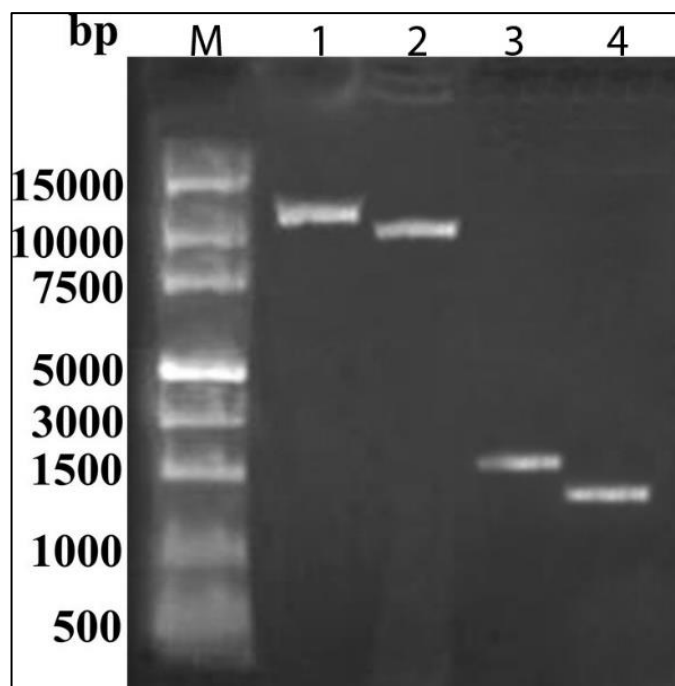


Figure 4.6: Image of recombinant plasmids in agarose gel after restriction enzyme digestions. M: marker, Lane 1: *ssexo-pg1* (~1865 bp) and pPIC9K (~3195 bp), Lane 2: circular pPIC9K -*ssexo-pg1*, Lane 3: circular pPIC9K Lane 4: *ssexo-pg2*.

4.2.2. Sequence Analysis

The presence of *ssexo-pg* gene constructs ligated into recombinant plasmids and their status within the plasmid (whether they are ligated towards the plasmid) were compared with the hypothetical gene (GenBank accession number: KXX78172.1) by DNA sequence analysis. As a result of sequence analysis, the presence, and correct ligation of both gene constructs in plasmids were determined.

4.3. Transformation of Plasmid into *Pichia pastoris*

4.3.1. Linearization of Plasmid and Electroporation

After recombinant plasmids were transferred to *Pichia pastoris* GS115 cells by electroporation, colony development was observed 7 days later in YPDS agar medium containing Geneticin (100 µg/mL). During this period, while colony development was not observed in the control group, colonies containing *ssexo-pg* gene structures were identified (Figure 4.7).

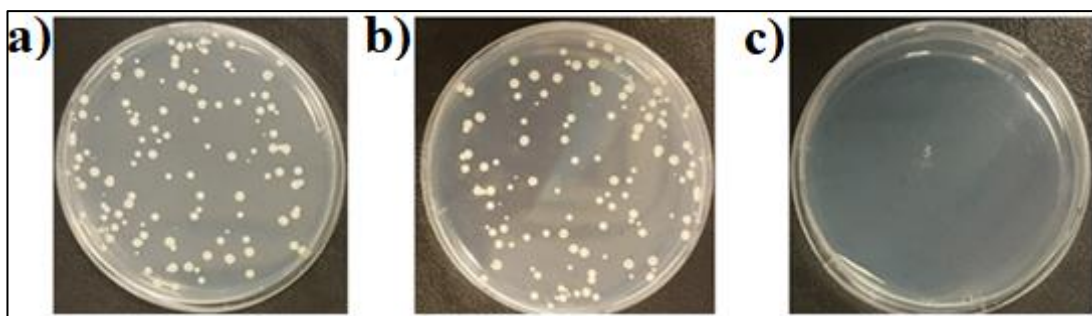


Figure 4.7: *P. pastoris* GS115 colonies developing on YPDS agar medium as a result of transformation. (a) Colonies containing the *ssexo-pg1* gene constructs (b) Colonies containing the *ssexo-pg2* gene constructs (c) Control: wild type *Pichia pastoris* GS115.

4.3.2. Selection of Transformed Cells

The commercially synthesized and codon-optimized *ssexo-pg1* and *ssexo-pg2* gene construct coding SsExo-PG in *P. pastoris* were successfully ligated into pPIC9K by confirming the sequence analysis. However, only *P. pastoris* GS115 colonies having *ssexo-pg2* plasmid exhibited protein expression of the enzyme. Therefore, further studies were continued with colonies having *ssexo-pg2* construct, hereafter referred to as *ssexo-pg*.

4.3.3. Optimum Conditions of Expression

Induction conditions for the best recombinant *P. pastoris* GS115 were determined as 1% methanol after 3 days at 30 °C by following *ssexo-pg* expression levels using SDS-PAGE (Figure 4.8).

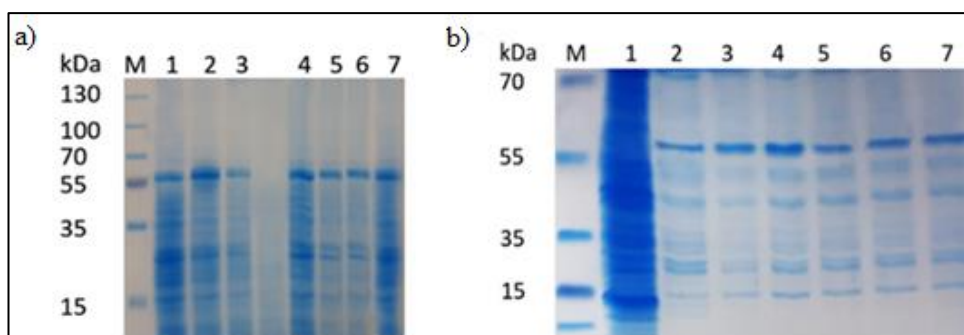


Figure 4.8: Optimization of *ssexo-pg* expression level. (a). Methanol concentration and incubation temperature; 1: Induction with 1.5% methanol, 2: Induction with 1% methanol, 3: Induction with 0.5% methanol 4: Incubation at 30 °C, 5: Incubation at 28 °C, 6: Incubation at 24 °C, 7: Incubation at 22 °C. (b). Time; 1: No methanol induction, 2: 24 h induction with 1% Methanol, 3: 48 h induction with 1% methanol, 4: 72 h induction with 1% methanol, 5: 96 h induction with 1% methanol, 6: 120 h induction with 1% methanol, 7: 144 h induction with 1% methanol.

4.4. Protein Purification

The recombinant SsExo-PG was purified from the recombinant *P. pastoris* GS115 cultures grown in BMMY medium under the optimized induction conditions. SDS-PAGE showed that the amount and level of purity of the SsExo-PG was enough for further studies (Figure 4.9). Change of total and specific enzyme activity and level of refinement during the purification process are outlined in Table 4.1. The results suggested that the Ni²⁺-NTA chromatography of the supernatant led to 11.84-fold purification and 21.6 mg/L SsExo-PG enzyme in the laboratory flask. The specific activity of the purified SsExo-PG enzyme was calculated as 184.8 U/mg.

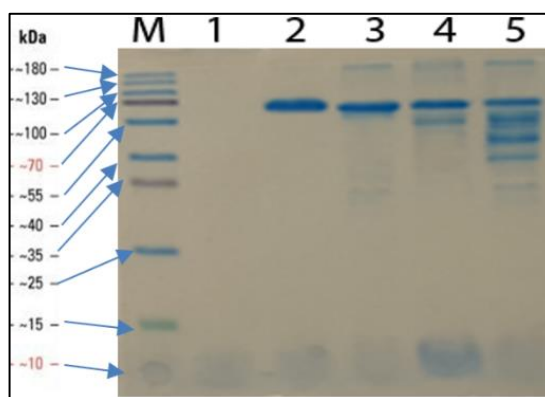


Figure 4.9: SDS-PAGE analysis image of purification steps of SsExo-PG expression. M: Marker 1: Buffer B 2: Concentrated 3: Elution with 400 mM Imidazole 4: Elution with 100 mM Imidazole 5: Supernatant.

Table 4.1: Purification factors and activity recovery of SsExo-PG during the different steps of protein purification.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold Purification	Activity yield (%)
Supernatant	255.8	1401	10.71	1.0	100
Ultrafiltration	112.4	1142	28.94	2.27	81.51
Ni ⁺ -NTA	21.6	944	184.8	11.84	67.38

4.5. Biochemical Characterization Analysis

4.5.1. Effect of pH, Temperature, Metal Ions and Solvents on Activity

The activity of the purified SsExo-PG was assayed for optimization of the different parameters. SsExo-PG is an acidic enzyme since pH 4 was identified as the optimum value for the highest activity (Figure 4. 10a). However, the enzyme started to lose activity after pH level exceeded 4. The optimum temperature for the SsExo-PG activity was found to be 60 °C (Figure 4. 10b). The enzyme lost its activity completely at 90 °C.

While 10% methanol and isopropanol-improved enzyme activity, the rest of the organic solvents used inhibited the activity of the enzyme (Figure 4.10c). Cationic metals had different effects on SsExo-PG activity depending on the concentration. Mg²⁺, Mn²⁺, and Zn²⁺ did not have a serious effect on the SsExo-PG activity, but the presence of Ag²⁺ led to an approximately 60% decrease inactivity. Fe²⁺ led to a loss of about 90%. Cu²⁺ increased about 20% relative activity of SsExo-PG (Figure 4.10d).

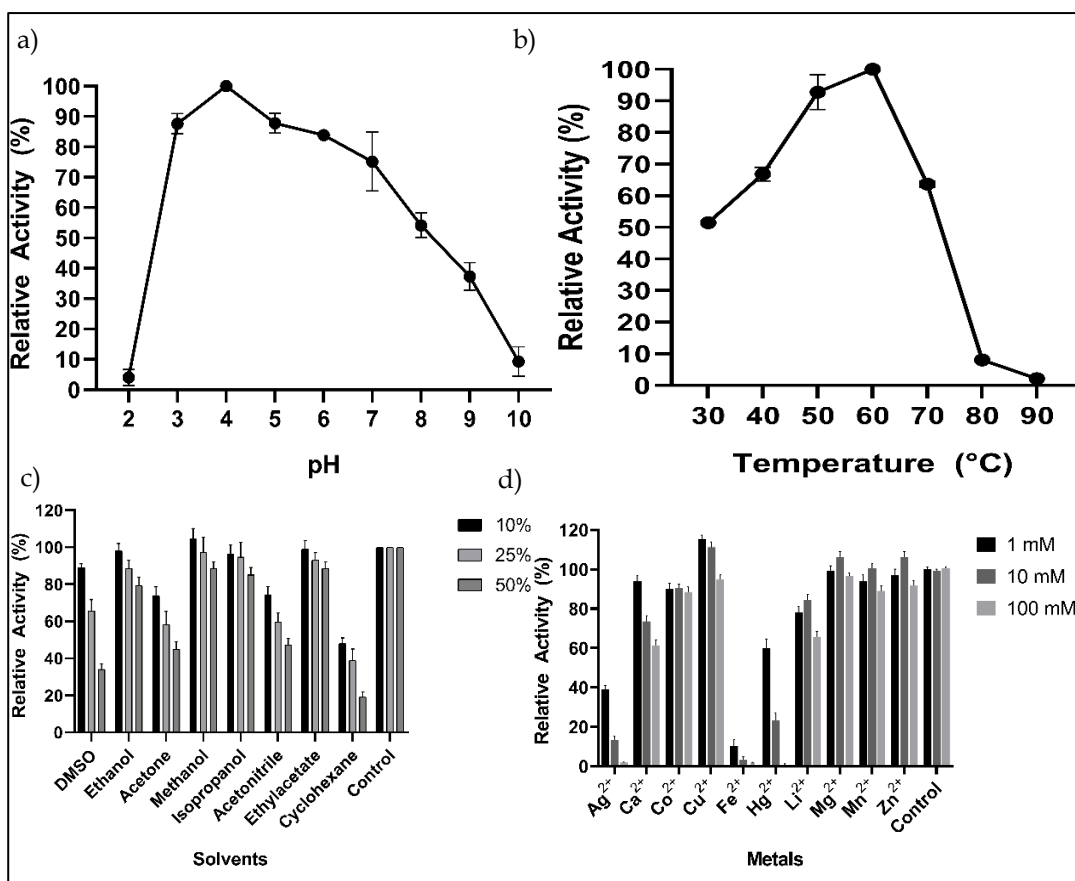


Figure 4.10: The effect of (a) pH (b) temperature (c) solvents (d) metals, on the relative activity of SsExo-PG. All Values are based on the Mean $\pm$ SD of three individual observations.

4.5.2. Storage Stability Assay

The optimum storage temperature was determined to be +4 °C. In contrast to the loss of all activity at -20 and -80 °C, the enzyme kept up to 40% of its original activity after 8 weeks at 4 °C (Figure 4.11).

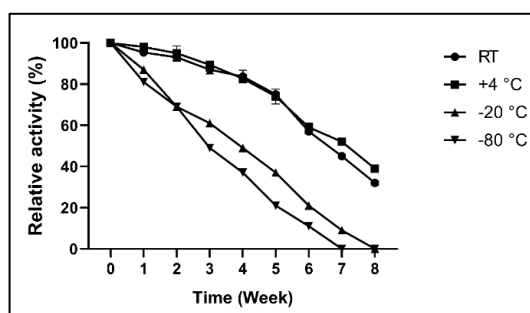


Figure 4.11: The results of the storage effect on SsExo-PG relative activity. All Values are based on the Mean $\pm$ SD of three individual observations.

4.5.3. Kinetic Assay

The K_m and k_{cat} values for PGA were calculated to be 0.58 μM and 179 s^{-1} , respectively (Table 2).

Table 4.2: Kinetic parameters of SsExo-PG and the selected Exo-PGs.

Organism	PGA			Ref.
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)	
<i>S. schenckii</i> 1099-18	0.58	179	308.62	This thesis
<i>Aspergillus flavus</i> MTCC 7589	2.08	96	46.15	[77]
<i>Penicillium janthinellum</i> sw09	1.74	301.33	173.17	[2]
<i>Penicillium janthinellum</i> VI2R3M	2.56	277	108.20	[84]
<i>Aspergillus fumigatus</i> MTCC 2584	2.4	44	18.33	[85]
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	0.67	21	31.34	[86]
<i>Phanerochaete chrysosporium</i>	2.5	440	176	[87]
<i>Aspergillus sojae</i>	0.424	-	-	[88]
<i>Penicillium notatum</i>	16.6	-	-	[5]
<i>Rhizopus oryzae</i>	55	10.3	0.18	[89]
<i>Aspergillus giganteus</i>	3.25	770	236.92	[90]
<i>Galactomyces citri-aurantii</i> IJ-1	0.94	2881.6	3065.53	[91]

4.6. Structural Analysis

4.6.1. Deglycosylation and MALDI-TOF MS Analysis

The structure of SsExo-PG was analyzed by using computer software and laboratory experiments. SsExo-PG was determined as a single band between 56-70 kDa using gel electrophoresis of protein (Figure 4.12a). The molecular weight of SsExo-PG was 56.21 kDa (Figure 4.12b) without the glycosylation site using the MALDI-TOF MS chromatogram and correlated well with the theoretically calculated Mw (50.68 kDa) and approximate molecular mass of 55-70 kDa. The two peaks of the MALDI-TOF MS also proved SsExo-PG digestion of EndoH resulted in releasing the glycosylation site (Figure 4.13).

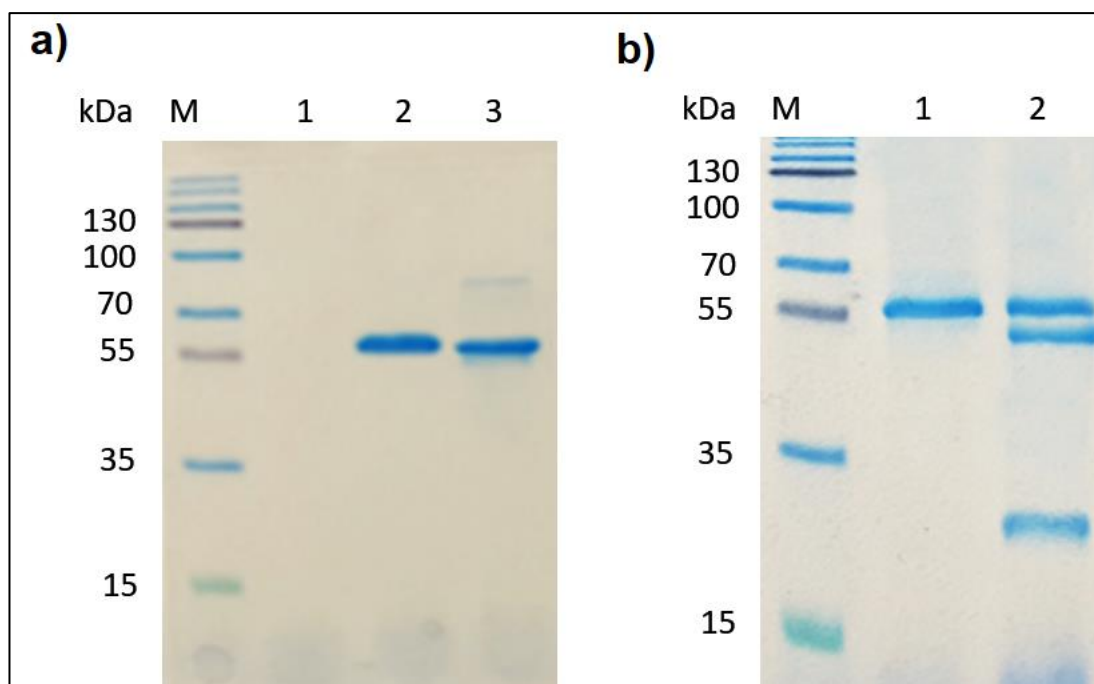


Figure 4.12: SDS-PAGE analysis of SsExo-PG. (a) Purified and concentrated SsExo-PG; M: Marker, Lane 1: Wash buffer, Lane 2: Concentrated and Purified SsExo-PG, Lane 3: SsExo-PG after purification. (b) Deglycosylation of purified SsExo-PG; Lane 1: Purified SsExo-PG, Lane 2: Deglycosylated SsExo-PG with EndoH treatment.

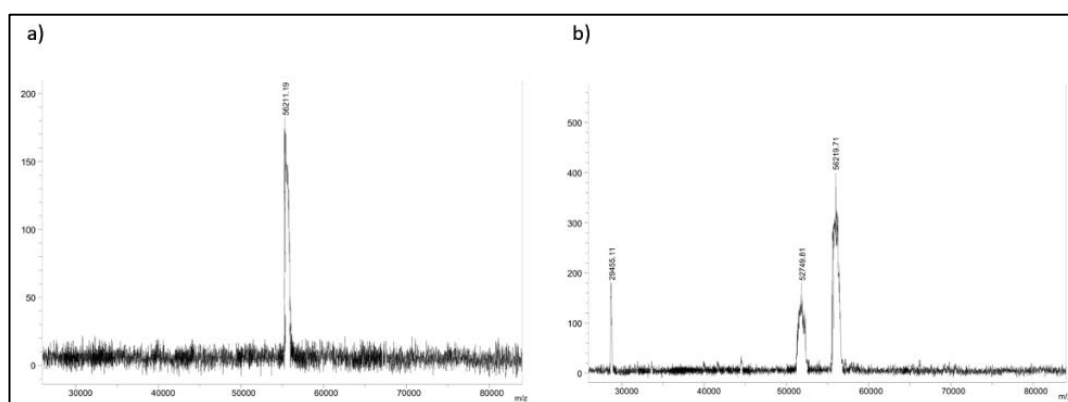


Figure 4.13: MALDI-TOF mass spectrum of the purified SsExo-PG. (a) Purified SsExo-PG with glycosylation site (56.21 kDa) (b) Deglycosylated SsExo-PG (52.74 kDa).

4.6.2. Homology Modeling and Molecular Docking Studies

A 3D homology model of SsExo-PG was created using I-TASSER [78] and *Aspergillus tubingensis* endo-xylogalacturonan hydrolase (XghA, PDB ID: 4C2L) as a template with 38.08% identity. Despite SsExo-PG and XghA having low sequence similarity (38%), the overall shape of the substrate-binding pocket and active site

residues of the two enzymes were conserved, similar to the results in previous studies [2, 88, 92] (Figure 4.14a).

The substrate PGA was bound in the active site through H-bonds with Tyr207 (OH-2.5 Å and HH-2.1 Å), Asp248 (OD1-2.8 Å), Asp267 (OD2-2.3 Å), and Tyr371 (HH-1.5 Å) (Figure 4.14b).

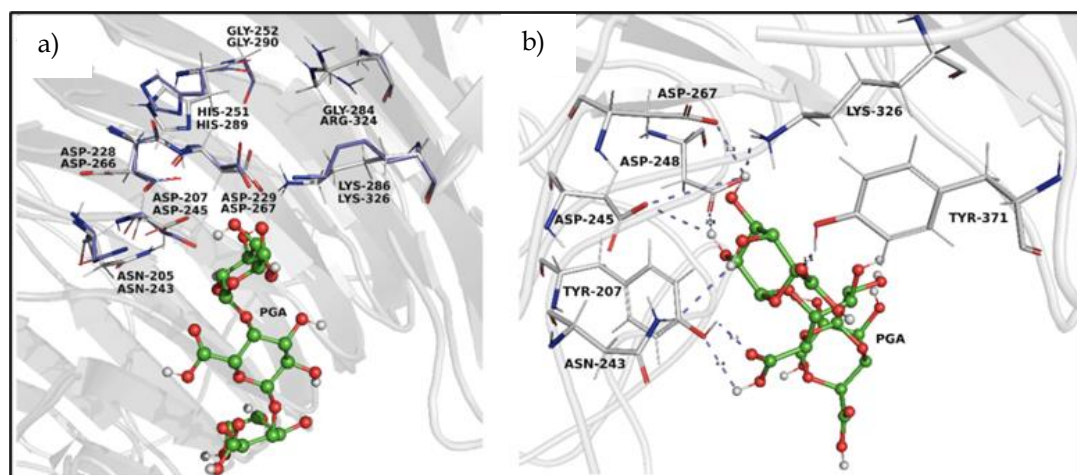


Figure 4.14: The homology model of SsExo-PG is superimposed with the structure of XghA (PDB ID: 4C2L). (a) Active site comparison of SsExo-PG (cyan) and XghA (magenta), (b) the active site of the SsExo-PG enzyme, docked PGA substrate, and SsExo-PG homology model shown in cyan.

Five disulfide bridges are present in the SsExo-PG structure: Cys47-Cys70 (1), Cys268-Cys285 (2), Cys370-Cys379 (3), Cys413-Cys419 (4), and Cys440-Cys452 (5). It has been suggested that Cys230 directly follows the two catalytic aspartates Asp266 and Asp267 and is responsible for ensuring the correct local folding [93]. The substrate-binding residue Tyr371 (Figure 4.14) may be stabilized by the one partner of the third disulfide bridge, Cys370 [79]. Protein engineering studies have demonstrated that Asp245, Asp266, and Asp267 are precisely involved with catalysis, whereas Lys326 plays a role in substrate binding affinity and Arg324 is essential for the proper orientation of the substrate [94, 95].

The secondary structure prediction of the SsExo-PG amino acid sequence (Figure 4.15a) and 3D homology model-docked PGA substrate is represented (Figure 4.15b).

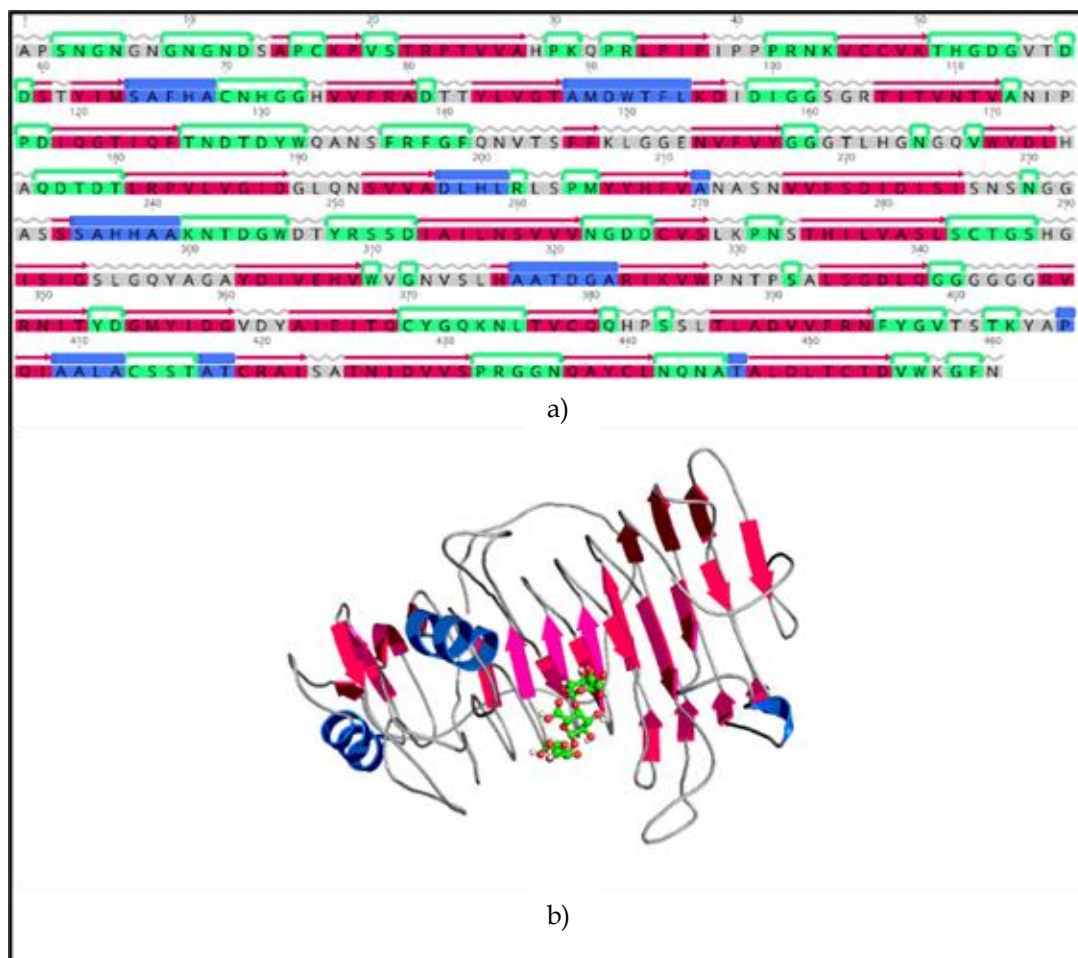


Figure 4.15: (a) Protein secondary structure prediction; (b) Cartoon representation of 3D homology model belong to SsExo-PG. Residues in α -helices, β -strands, loops, and turns are highlighted as blue cylinders, pink arrows, grey coils, and green elbow arrows, respectively.

4.6.3. Thin Layer Chromatography Analysis

Products hydrolyzed from the enzymatic reaction on PGA and 74% esterified pectin were analyzed using Thin-Layer Chromatography analysis. PGA digestion experiment showed that the only observed product was mono-galacturonic acid while di-galacturonic acid and tri-galacturonic acid could not be observed (Figure 4.16).

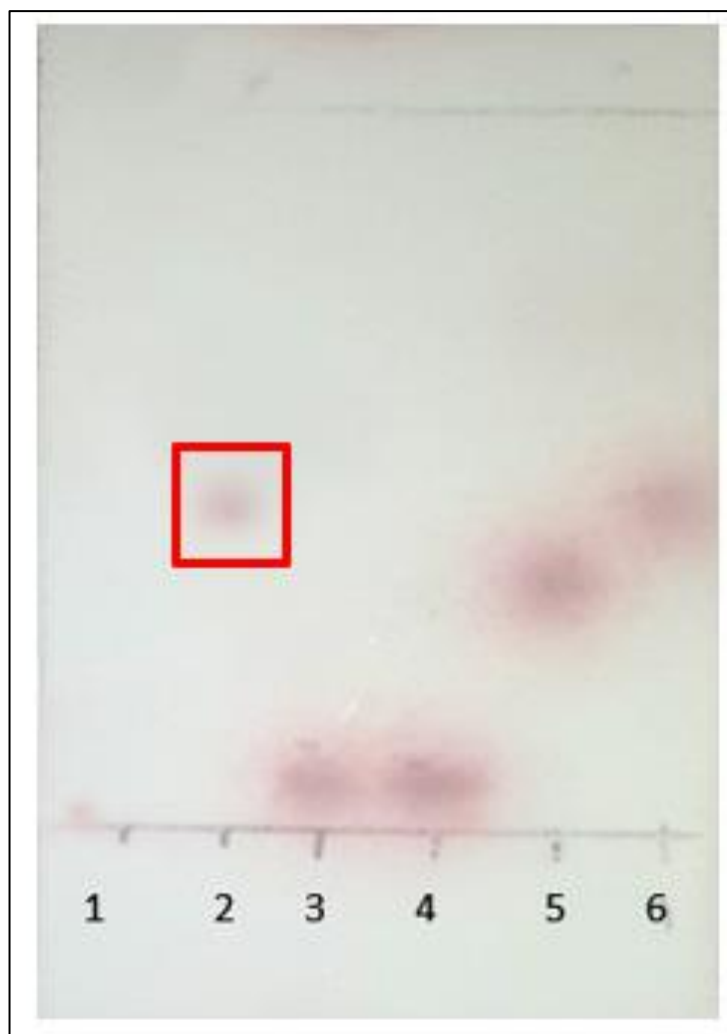


Figure 4.16: TLC analysis of the products obtained from the enzymatic reaction of SsExo-PG. Lane 1: The reaction mixture contained 74% esterified pectin Lane 2: and 2.0 mg/mL of PGA Lane 3: in acetate buffer (pH 4.5); trigalacturonic acid Lane 4: PGA Lane 5: digalacturonic acid Lane 6: monogalacturonic acid. The dot in the red square indicates monogalacturonic acid as the main hydrolysis product of SsExo-PG on PGA.

4.7. Apple Juice Clarification Assay

All clarification performances were expressed as NTU units. It was seen that SsExo-PG had a remarkable effect on the clarity of apple juice (Figure 4.17). The efficiency of SsExo-PG and its mixtures compared to its commercial counterparts in apple juice clarification were evaluated (Figure 4.17). The SsExo-PG treatments were shown to significantly reduce relative viscosity in apple juice, with SsExo-PG reducing turbidity up to 50% when used at a concentration of 15 mg/L. However, turbidity decreased less when the Enerzyme HT and SsExo-PG 15 mg/L combination

were used. SsExo-PG was shown to be the most effective at a concentration of 30 mg/L rather than 45 mg/L.

The turbidity of the apple juice decreased by 50% when incubated with SsExo-PG at 15 mg/L, by approximately 70% when incubated with SsExo-PG at 30 mg/L, and by approximately 65% when incubated with SsExo-PG at 45 mg/L (Figure 4.18).

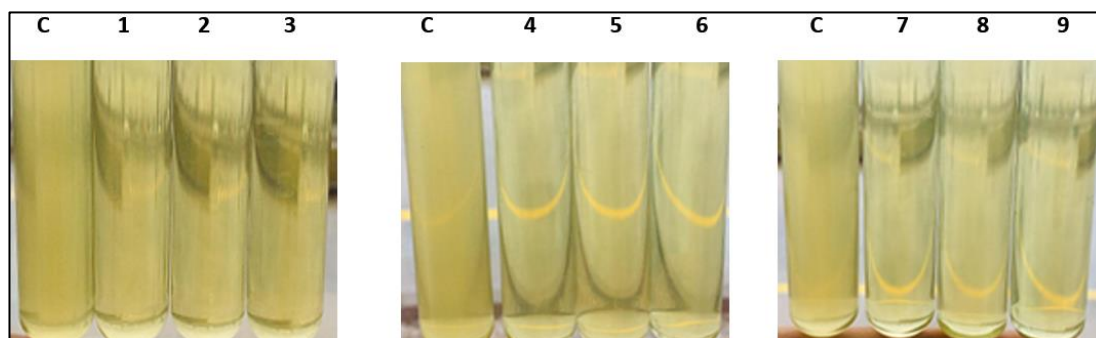


Figure 4.17: Clarity performance of enzyme recipes. C: Control as apple juice after pasteurization at rinsing stage. 1: Enerzym HT (36 mg/L) + Fruktozym P6L (24 mg/L) + Fruktozym Flux (19 mg/L), 2: Enerzym HT (36 mg/L) + Fruktozym P6L (24 mg/L), 3: Enerzym HT (36 mg/L) + Fruktozym Flux (19 mg/L), 4: Enerzym HT (36 mg/L) + SsExo-PG (15 mg/L), 5: Enerzym HT (36 mg/L) + SsExo-PG (30 mg/L), 6: Enerzym HT (36 mg/L) + SsExo-PG (45 mg/L), 7: SsExo-PG (15 mg/L), 8: SsExo-PG (30 mg/L), and 9: SsExo-PG (45 mg/L).

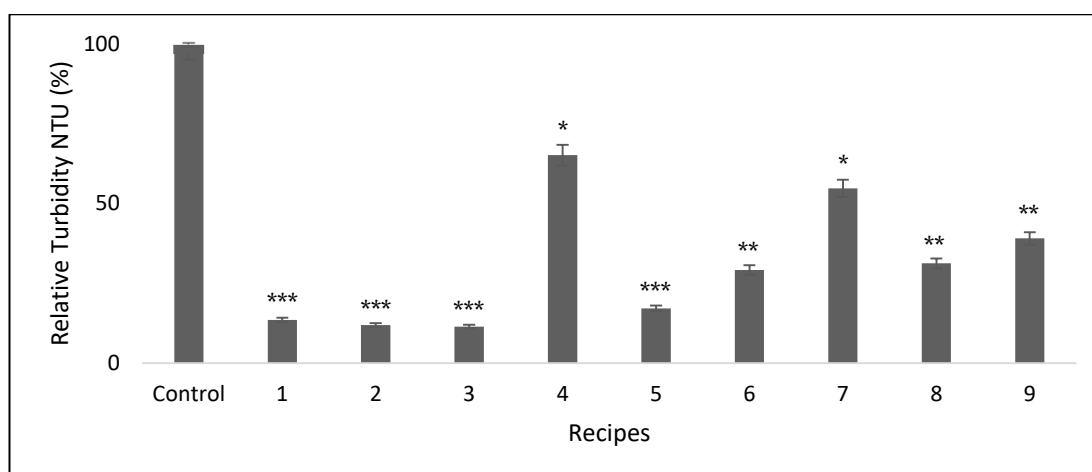


Figure 4.18: Relative turbidity (%) values of various enzyme recipes. Control: Apple juice after pasteurization at rinsing stage 1: Enerzym HT (36 mg/L) + Fruktozym P6L (24 mg/L) + Fruktozym Flux (19 mg/L), 2: Enerzym HT (36 mg/L) + Fruktozym P6L (24 mg/L), 3: Enerzym HT (36 mg/L) + Fruktozym Flux (19 mg/L), 4: Enerzym HT (36 mg/L) + SsExo-PG (15 mg/L), 5: Enerzym HT (36 mg/L) + SsExo-PG (30 mg/L), 6: Enerzym HT (36 mg/L) + SsExo-PG (45 mg/L), 7: SsExo-PG (15 mg/L), 8: SsExo-PG (30 mg/L), and 9: SsExo-PG (45 mg/L). All experimental parameters were compared to control group.

5. DISCUSSION

Heterologous expression in *P. pastoris* has been used in food processing for decades because of its high yield protein production and PTM properties. The pectinolytic enzyme SsExo-PG from *S. schenckii* 1099-18 was successfully expressed heterologously in *P. pastoris*. This thesis describes the detailed biochemical and structural characterization of SsExo-PG. Because of its prominent exo-cleaving property and high primary structure similarity, SsExo-PG was found as a member of the family hydrolyze 28 Exo-PG [96]. Although several Endo-PGs having exo-cleaving characteristics, several of them named Endo-PG since their sequence similarity to Endo-PGs [96].

Secretion signal peptide preference is significant and necessary for the effective heterologous expression of proteins in *P. pastoris*. Recent studies have examined various signal peptides have been examined [87, 97-99]. For example, it has been observed that the α -factor secretion signal showed higher protein expression yield than the native signal when expressing endo- β -1,4-glucanase [100]. Also, monocyte chemoattractant protein-1 has been observed to be expressed at the same level with either the native signal sequence or the α -factor secretion signal [101], and the same expression level has been obtained using the native signal or α -factor secretion signal while expressing human monocyte chemoattractant protein-I [102]. Using a native signal peptide affected the expression level by increasing alkaline protease production 1.5 times higher than using the α -factor secretion signal [97].

The construct without the native signal peptide and detection of pectinolytic activity in the medium fraction and not in the cell fraction supported our belief that SsExo-PG is signal showed higher protein expression yield than the native signal when expressing endo- β -1,4-glucanase [100]. Also, monocyte chemoattractant protein-1 has been observed to be expressed at the same level with either the native signal sequence or the α -factor secretion signal [101], and the same expression level has been obtained using the native signal or α -factor secretion signal while expressing human monocyte chemoattractant protein-I [102]. Using a native signal peptide affected the expression level by increasing alkaline protease production 1.5 times higher than using the α -factor secretion signal [103].

The construct without the native signal peptide and detection of pectinolytic activity in the medium fraction and not in the cell fraction supported our belief that SsExo-PG is extracellular, contrary to the majority of Exo-PGs examined [88].

PTM refers to the covalent and generally enzymatic modification of proteins following protein biosynthesis. For most proteins, these changes are one of the last steps in protein biosynthesis [104]. Glycosylation is one of the most common PTM of proteins and has been described in numerous fungal PGs, such as *Aspergillus niger* [105, 106] *B. cinerea* [106], *K. marxianus* [107], and *S. purpureum* [108].

SDS-PAGE analysis was used to assess the molecular weights of the SsExo-PG, confirming that SsExo-PG has molecular weights between 53 and 58 kDa (Figure 4.12a). However, the existence of multiple bands identified after treatment with an EndoH enzyme had different molecular weights. Consequently, when we predicted the possible glycosylation of the SsExo-PG, we observed two bands after treatment with EndoH (Figure 4.12b). Thus, purified SsExo-PG can be post-translationally modified by glycosylation, and the multiple smeared bands were assumed to be glycosylated.

The experimental and computational studies illustrated the structural properties of SsExo-PG. MALDI-TOF MS results confirmed that SsExo-PG is a 56.21 kDa monomer (Figure 4.13a), nearly 6 kDa larger than the molecular mass calculated from the amino acid sequence. The large mass incompatibility indicated the presence of PTMs on SsExo-PG, and the detected broad peak and poor mass resolution in the MALDI-TOF spectrum were suggestive of heterogeneous glycoforms. Therefore, the intact protein was deglycosylated by EndoH directly on the MALDI target to confirm the existence of N-linked glycosylation. A new peak appeared at about 4 kDa, less than the intact protein peak, indicating that the recombinant SsExo-PG was modified by N-linked glycans after 60 minutes of digestion (Figure 4.13b).

Figure 4.14 shows the predicted substrate-binding residues in SsExo-PG, which are Tyr207, Asn243, Asp245, Asp248, Asp267, Lys326, and Tyr371, indicating that the enzyme belongs to glycoside hydrolase family 28 (GH28). Molecular-modelling and docking studies revealed that the substrate-binding pockets of XghA and SsExo-PG are formed by residues Asn205-243, Asp207-245, Asp228-266, Asp229-267, His251-289, Gly252-290, Gly284-Arg324, and Lys286-326, which are strictly conserved among bacterial-, fungal-, and plant-originated PGs [88, 109]. However,

active site-forming amino acid positions are slightly different (Figure 4.14). This diversity may have a role in supplying tolerable kinetic efficiency to SsExo-PG (Table 2).

pH can distribute the charge on the enzyme thus, substrate binding and catalytic reactions can be affected directly [110]. The influence of varying pH (from 2.0 to 10) on the catalytic activity of SsExo-PG was determined and the results are presented in Figure 4.10. SsExo-PG showed good activity at pH 3.0-6.0 with an optimum at pH 4.0. The activity findings based on pH were following earlier studies, i.e., the purified PG enzyme from *Aspergillus niger* AN07 was active at a pH range of 4.0-7.0 [111-113]. pH 5.0 and 5.5 were reported as optima for the best function from *P. viridicatum* and *A. niger* Exo-PG respectively, was found to be optimal. Another study found that *A. niger* PG has the highest activity at pH 4.8 and stability at a wider pH array of 4.0 to 5.5 [114]. There are also Exo-PGs that have a wide-range of pH tolerance regarding enzyme activity [5, 115, 116].

The optimum temperature of SsExo-PG was found to be 60 °C, and the enzyme activity of SsExo-PG was largely reduced at the higher temperatures because of protein denaturation; activity terminated at 90 °C. The Exo-PG produced from *F. lycopersici* exhibited the highest activity at 50 °C [117] and, likewise, *L. gongylophoru* Exo-PG reached maximum activity at 60 °C [118]. The maximum temperature for Exo-PG produced by *P. sanguineus* was 80 °C [119]. SsExo-PG was found to be active between 30-60 °C that is apple juice clarification process temperature [120].

Some biotechnological applications need tolerance to several factors like organic solvents. Pectinases that have such tolerance to organic solvents bring powerful catalytic property. Cyclohexane and acetonitrile inhibit activity by destroying the structure of the enzyme, while the enzyme retains maximum activity in methanol, isopropanol, and ethyl acetate. Different solvents have also been reported to affect enzyme activity: When treated with Tween-80 and Triton X-100, PG from *S. thermophile* was faintly inhibited. Also, SDS was shown to have a strong inhibitor effect that terminated the enzymatic activity of PG from *S. thermophile*, possibly because of the protein denaturation effect of SDS. β -Mercaptoethanol was found to be a strong inhibitor of PGs [15]. Disulfide linkages play an important role in the conformation of proteins; β -Mercaptoethanol can destroy disulfide bridges, so

treatment with β Mercaptoethanol can terminate enzymatic activity. This thesis is very important for understanding organic solvents' effects on Exo-PGs because it is more comprehensive than any other in the current literature.

Metal ions have various effects on enzymes. Some can inhibit enzymatic activity while others boost activity [121]. Enzymes containing one or more metals, known as metalloenzymes, cannot catalyze reactions without the essential ion at the active site. Metal salts also contribute to improved activity by inducing electrostatic interactions that fold the protein into the shape necessary for its optimal performance [122, 123]. Therefore, the influence of various metal ions, including Ag^{2+} , Ca^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Hg^{2+} , Li^{2+} , Mg^{2+} , Mn^{2+} , and Zn^{2+} on SsExo-PG activity was assessed (Figure 4.10). The results indicated that Cu^{2+} significantly enhanced enzyme activity. Notably, the metal ion Mn^{2+} was the strongest inducer of Exo-PG, which is in agreement with recent studies [5, 112]. In contrast to the control, the supplementation of Mn^{2+} increased Exo-PG activity up to 32%, followed by Co^{2+} activity improvement of up to 19% at 5 mM concentrations [94]. Cu^{2+} may have interacted non-covalently with carboxylic groups of amino acids present in the enzyme active region, acting as a cofactor for enzyme functioning. The ions of iron and silver reduced SsExo-PG activity to about 10%.

Several storage temperatures were used to determine effects on SsExo-PG activity (Figure 4.11). A storage temperature of +4 °C is slightly better than RT, but SsExo-PG maintained half its original activity in both temperatures, even after eight weeks. The enzyme was stored as purified and without any preservative (glycerol, DMSO, etc.) thus lower temperatures may have damaged protein folding. Therefore, it can be deduced that the enzyme displays remarkably high storage stability, which is a novel characteristic according to the published literature [92, 124].

TLC analysis of the products of PGA hydrolysis indicated that trigalacturonates and oligosaccharides were produced in the initial hydrolysis (15 min) and accumulated when the hydrolysis process was continued (Figure 4.16). SsExo-PG hydrolyze process resulted in only monogalacturonic acids, throughout the incubation period. Exo- and Endo-PGs utilize different hydrolytic modes of action. Endo-PGs hydrolyze randomly in connection with the polygalacturonate network, while Exo-PGs release galacturonic acid through the hydrolysis of the PGA [125].

This behavior is consistent with the indication that the action mode of SsExo-PG is exo-acting.

Adding pectinolytic enzymes, finning agents such as bentonite, or using filters and homogenization can be used for clarifying apple juice. Filters used in clarification can leave harmful residues, whereas adding chemicals may result in hazardous silica dust [126]. The negative consequences of using these materials are an incentive for the development of alternate processing strategies for juice production. Centrifugation is widely employed for processing apple juice and allows for semi-continuous operation. Usually, the maximum force of 1000g is used for a short time [127]. However, centrifugation requires excessive energy and cost.

Fresh apple juice has a pH range between 3.5 and 4.0 [128]. Experiments in this thesis showed that the optimum reaction pH of SsExo-PG correlates with the pH of fresh apple juice that introduces a big challenge to easily adopt the enzyme to the clarification process. A single study tested the clarification performance of Exo-PGs in fresh apple juice using *Penicillium notatum* [5]. However, in that thesis, the reported NTU values for apple juice clarification were not obtained from the in-process application. The amount of enzyme used, and the details of the processing conditions were not clear, and the reported NTU rates were obtained by correlating the measured spectroscopic data [5]. We compared SsExo-PG and the widely used commercial enzymes by testing their clarification performance with different treatments in this thesis.

A 30 mg/L concentration of SsExo-PG clarified better than a 45 mg/L concentration of SsExo-PG. Using much more protein may result in aggregation that inhibits enzyme activity [129]. SsExo-PG may reduce the viscosity and heighten the light transmittance of apple juice. This thesis showed SsExo-PG to be slightly more effective than other commercial enzyme mixtures. Although the combination of fructose, amylase, and cellulase activity of the commercial enzymes, SsExo-PG is still superior as a single Exo-PG enzyme. This may be explained by either presence of starch or another colloid besides pectin or the requirement of the synergistic action of several enzymes together with PLs, other PGs, PEs, etc. [37]. Acidic SsExo-PG has been revealed as solvent tolerant and as having high storage stability at low temperatures even though it contains no preservatives. Thus, SsExo-PG is a promising enzyme for the clarification of apple juice when combined with other

pectinases or even another degrading enzyme (such as cellulose, fructose, or amylase).

Further studies should focus on increasing the specific activity and substrate specificity using protein engineering techniques [130]. Also, investigation of new mixtures that include commercial enzymes may increase the potential of the enzyme for use in not only food production but also in textile and waste management processing. Different immobilization techniques of enzymes in industrial implementation can improve activity, stability, specificity, purity of the enzyme, and more [131-135].

6. CONCLUSION

Pectinolytic enzymes are widely used in different biotechnological processes. The objective of this thesis was to heterologous SsExo-PG production using *P. pastoris* expression. SsExo-PG was purified and widely characterized in terms of biochemistry and computational structure modeling. The optimum activity condition values of SsExo-PG were determined. Kinetic values and effects of solvents and metal ions were also determined, contributing to the literature on Exo-PG. The clarification performance of SsExo-PG compared with commercial tested pectinolytic enzymes shows it to be slightly better. This thesis suggests the new recombinant Exo-PG should be produced on a large-scale using fermentation technology, as engineering and immobilization of the enzyme may contribute to more efficient, low-cost, and eco-friendly applications.

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BIOGRAPHY

Ersin was born in Erzurum, Turkey, on 1988. He received his B.Sc. Degree in Biology from Anadolu University (a.k.a Eskişehir Technical University) in 2011. He started to Biotechnology M.Sc. program from Ege University in 2011. He graduated in 2015 from M.Sc. after switching to Biotechnology M.Sc. program from Bezmialem Vakıf University. He worked as research assistant in Medical Biochemistry at Bezmialem Vakıf University.

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APPENDICES

Appendix A: Standard curve for DNS protocol.

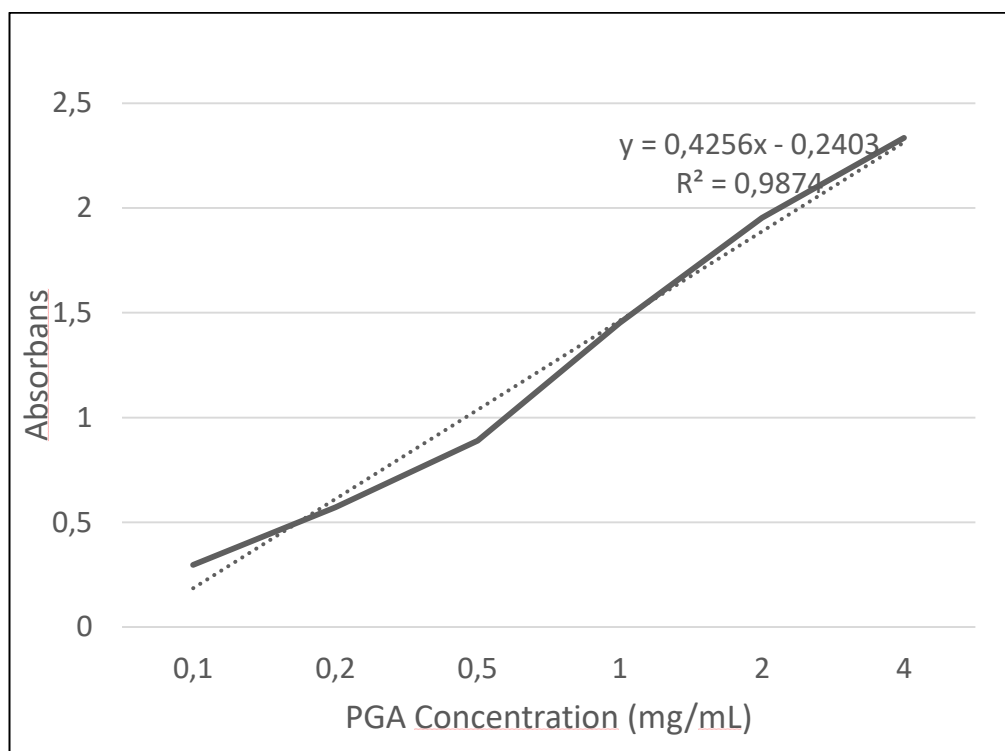


Figure A1.1: Standard curve for DNS protocol.

Appendix B: Publications related to the thesis

Karataş, E, Tülek A., Binay B., (2020) “From secretion in *Pichia pastoris* to application in apple juice processing: Exo-polygalacturonase from *Sporothrix schenckii* 1099-18” *Protein and Peptide Letters*, 28, 2

Karataş E., Tülek A., Tükel, S, Yildirim D., Binay B. "Encapsulation of *Sporothrix schenckii* 1099-18 exo-polygalacturonase in Fe₃O₄-hollow silica Shell composites" II. International Enzyme & Bioprocess Days, O-22, Kocaeli, Turkey, 01-03 September 2020.

Karataş E., Tülek A., Çakar M. M., Tamtürk F., Aktaş F., Binay B., “Characterization of a new Exo-polygalacturonase from *Sporothrix schenckii* 1099 18 in *Pichia pastoris*” II. International Enzyme & Bioprocess Days, O-78, Kocaeli, Turkey, 01-03 September 2020.

Karataş E., Tülek A., Binay B., “Heterologous Expression of a New ExoPolygalacturonase from *Sporothrix schenckii* 1099-18 in *Pichia pastoris*” 2th Eurasia Biochemical Approaches & Technologies (EBAT) Congress, O-74, Antalya, Turkey, 26-29 October 2019.